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GENETIC ENGINEERING OF POTATOES FOR DISEASE RESISTANCE

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Genetic Engineering of Potatoes for Disease Resistance

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Executive Summary

The objective of this project was to use gene transfer technology to increase resistance to viral and fungal diseases in potato cultivars of importance to Saskatchewan's seed industry. Novel traits or lines generated by this program may be protected under Plant Breeder's Rights Legislation and licenced exclusively to Saskatchewan's seed potato growers, thereby providing the industry with a distinct marketing advantage. ***Major advances achieved as a result of this project include the development of a method for the insertion of value added traits into plants that is both consumer friendly and free of patent restrictions. Additional achievements include the transferring of disease resistance genes for verticillium wilt, late blight, and the major viruses into several potato varieties.***

In tomato, the Ve disease resistance gene is implicated in race-specific resistance to infection by Verticillium species. Characterization of the Ve locus involved positional cloning and isolation of two closely linked inverted genes. ***Expression of individual Ve genes in susceptible potato plants conferred resistance to an aggressive race 1 isolate of Verticillium albo-atrum. Related genes have been isolated for late blight resistance. Studies of the primary structure of Ve1 and Ve2 suggest that the Ve genes encode a novel class of cell surface glycoproteins that can be used as a natural reporter to facilitate commercialization of transformed plants. Patents have been filed for the Ve genes.***

In collaboration with the Max Planck Institute, transgenic potato plants expressing mutant alleles of PLRV ORF4, the gene for the movement protein pr17 of the potato leafroll luteovirus, were generated in an effort to achieve broad-range protection against virus infection. Potato lines accumulating N- or C-terminally extended PLRV pr17 mutant proteins were ***resistant to infection by PLRV and the unrelated potato viruses PVY and PVX.*** *Full-length infectious cDNA clones of Canadian ($PLRV_{fle}$) and German ($PLRV_{flg}$) potato leafroll polerovirus isolates were constructed and antibodies directed against different domains were applied to the identification of previously unknown genes and proteins such as P1-C25. These genes and products are involved in essential aspects of viral replication and* ***provide novel strategies for the development of effective virus resistance.***

Project Background

The cornerstone of the North American commercial potato industry is the availability of reasonably priced, high quality seed. The Province of Saskatchewan has developed a reputation for production of seed potatoes of unparalleled quality, resulting in a doubling of the acres in seed production in each of the last four years. In part, the quality of Saskatchewan seed is a function of the northern growing environment, with long sunny days, short growing season and limited rainfall, combining to produce physiologically vigorous seed. The prairie environment is also not conducive to the development or spread of many of the diseases which can reduce seed quality and yield potential. However, many diseases still represent challenges to the Canadian potato industry.

Viral diseases such as Potato Leaf Roll (PLRV) and Potato Viruses X, Y and S represent serious threats to the seed and commercial potato industries. These diseases are spread by insects and by planting infected seed. Infection results in significant losses in yield and quality in the commercial crop. The regulatory system imposes very strict tolerances for levels of these viral diseases in the seed system. To meet these stringent tolerances, seed growers rely on costly roguing programs coupled with pesticide spray programs designed to eliminate the insect vectors. These efforts are costly, only marginally effective and have significant negative impact on both the environment and the health of the applicator.

The concept of generating sustainable viral resistance through transfer of virus coat protein genes is well established in many crops, including potatoes (Wilson 1993). Researchers at the Lethbridge Research Centre (AAFC) have developed Russet Burbank potatoes expressing the coat protein gene for PLRV (Kawchuk et al. 1990 and 1991). These potatoes are highly resistant to leaf rolling and show few of the tuber necrosis symptoms of this disease. Further, conferring resistance to the viral disease has not had any adverse effects on the agronomic or processing characteristics of this cultivar.

Potato foliage and tubers are also attacked by a range of fungal pathogens, both in the field and during storage. Growers have traditionally relied on fungicides to deal with these problems. The rising cost of these products, coupled with concerns about their toxicity in the environment and to consumers, has led the industry to seek alternatives. The recent emergence of strains of fungi resistant to previously effective control products (metalaxyl resistance in *Phytophthora infestans* and thiabendazole resistance in *Fusarium sambucinum*) has highlighted the shortcomings of excessive reliance on a limited range of control products.

A potential alternative to continuing dependence on chemical control strategies is to use plant transformation to enhance the potato crop's inherent ability to resist or tolerate attack by fungal pathogens. One of the most promising options is to transform the crop with genes that code for hydrolytic enzymes capable of selectively disrupting the cell wall constituents of the target fungal pathogens (Liu et al. 1994, Jongedijk et al. 1995).

Project Objectives:

AAFC Lethbridge, working in collaboration with the Department of Plant Sciences at the University of Saskatchewan and the Saskatchewan Seed Potato Growers Association, agreed to conduct the necessary trials to meet the following objectives;

1) To use the proven vector system and coat protein gene, developed by the Lethbridge Research Centre, to confer resistance to the Potato Leaf Roll Virus on a number of potato cultivars of commercial interest to Saskatchewan's seed industry.
2) Cloning, insertion and monitoring of coat protein genes for mosaics (potato viruses X and Y) and potato virus S in several important potato cultivars .
3) To improve the efficiency and efficacy of virus protection (PLRV, PVX, PVY and PVS) through genetic engineering of sub-gene constructs.
4) Cloning, insertion and monitoring of hydrolytic enzymes capable of protecting potatoes

against fungal attack.

5) To improve the efficiency and efficacy of fungal protection through genetic engineering of the genes coding for the hydrolytic enzymes.

6) Disease resistant materials generated by this program were to be protected under Plant Breeder's Rights Legislation and licenced exclusively to Saskatchewan's seed potato growers, thereby providing the industry with a distinct marketing advantage.

Progress to Project Completion Relative to Project Milestones:

Project Contractor	Activity	Timeframe	Status*
Leaf Roll Resistance	Transform locally important potato cultivars with the PLRV coat protein gene	97/98	C
	Confirm transcription and translation	98/99	C
	Challenge transformed plants with PLRV		
	- greenhouse trials	98/99	C
	- field trials	99/00	C
	Impact on agronomic performance	99/02	C
Virus X, Y and S Resistance	Clone coat protein genes	97/98	C
	Transform important potato cultivars	98/99	C
	Confirm transcription and translation	98/99	C
	Challenge transformed plants with viruses		
	- greenhouse trials	99/00	C
	- field trials	99/00	C
	Impact on agronomic performance	99/00	C

Subgene study	Prepare a range of subgene fragments of PLRV, PVX, PVY and PVS coat proteins	97/98	C
	Transform with standard gene and subgene fragments	98/99	C
	Compare levels of transformation, transcription and translation for subgene fragments	98/99	C
	Compare virus resistance for subgene fragments under greenhouse conditions	99/00	C
Fungal Resistance	Identify potential fungi hydrolytic enzymes	97/98	C
	Clone genes for hydrolytic enzymes	97/98	C
	Transform potatoes	98/99	C
	Monitor expression of novel genes	98/99	C
	Challenge transformed lines with 2 to 4 different fungi		
	- greenhouse trials	99/00	C
	- field trials	99/02	C
	Impact on agronomic performance	99/02	C
Enzyme Engineering	Engineering to enhance activity/efficacy of hydrolytic genes	99/00	C

*** O = ongoing, P = pending, C = completed**

Achievements

A. Resistance to Fungi

Objective A1 -Verticillium Wilt Resistance

Verticillium wilt is a common fungal disease that causes severe yield and quality losses in many crops including alfalfa, cotton, cucurbits, eggplant, mint, potato, tomato, strawberry, and sunflower (1). Several species of *Verticillium* have been reported to cause wilt and control has often relied on the use of expensive chemical fumigants that may adversely impact health and environment. In a few cases, effective control of verticillium wilt has been reported in specific crops that exhibit race-specific resistance (2, 3).

Plant resistance to viruses, bacteria, and fungi frequently involves specific host-pathogen interactions between the products of a plant resistance gene (*R*) and corresponding avirulence gene (*Avr*) in the pathogen (4, 5). Absence of either of these entities results in a susceptible response whereby plant defenses are not elicited and infection proceeds. Several plant disease resistance genes have been cloned and assigned to one of five classes based on structural features. One class includes R genes that encode a cytoplasmic serine/threonine protein kinase such as *Pto* in *Pseudomonas syringe* (6). Another class includes the *P. syringe RPS*2 and *RPM*1 resistance genes of *Arabidopsis* and the tomato *Fusarium oxysporum* resistance gene *I*2, which encode cytoplasmic proteins with a leucine zipper, nucleotide-binding site (NBS), and C-terminal leucine-rich repeat (LRR) (7-10). A third class of cytoplasmic proteins possess LRR and NBS motifs and an N-terminal domain with homology to the mammalian Toll/interleukin-1 receptor (TIR) domain. These include the tobacco *N* gene for resistance against tobacco mosaic virus, the flax *L*6 gene for resistance to *Melamspora lini*, and the *Arabidopsis RPP*5 resistance gene for *Peronospora parasitica* (11-13). A fourth class consists of the tomato *Cladosporium fulvum* resistance genes that have an extracellular LRR, a membrane spanning domain, and a short cytoplasmic C-terminus (14). The rice *Xa*21 resistance gene for *Xanthomonas* represents a fifth

class having an extracellular LRR and an intracellular serine/throenine kinase domain (15).

In tomato (*Lycopersicon esculentum*), resistance to race 1 of *Verticillium dahliae* and other species is conferred by a single dominant *Ve* gene that was mapped to linkage group IX (16). We previously identified in near-isogenic tomato germplasm a codominant random amplified polymorphic DNA (RAPD) marker within 3.2 ± 0.3 cM of *Ve* (17). Sequences of the RAPD were subsequently used to develop allele-specific sequence-characterized amplified regions (SCARs) determined by high resolution mapping to be within 0.67 ± 0.49 cM or 290 kb of *Ve* (18).

We describe the positional cloning of two inverted resistance genes from the tomato *Ve* locus and demonstrate that both *Ve*1 and *Ve*2 independently confer resistance to an aggressive race 1 isolate of *Verticillium albo-atrum* in potato. Structures within the *Ve* genes suggest they encode a novel class of cell surface glycoproteins with signals for receptor-mediated endocytosis and leucine zipper or PEST (P, Pro; E, Glu; S, Ser; T, Thr) sequences.

Screening of tomato genomic and cDNA libararies. Genomic clones (Fig. 1) were isolated by screening a λ EMBL3 library (Clonetech) of the *V. dahliae* race 1 resistant *L. esculentum* germplasm VFN8 initially with allele-specific SCARs (18). Approximately three copies of the genome represented by 2 x 10^5 recombinant plaques were transferred to duplicate Hybond N^+ membranes and probed. Rescued clones were subcloned into pBluescript SK(-) and sequenced.

A Stratagene cDNA cloning kit was used to prepare and unidirectionally clone cDNA as described by the manufacturer. Total RNA was isolated from detached leaves of greenhouse propagated verticillium wilt resistant *L. esculentum* cultivar Craigella, stressed in 1 mM L-serine for 48 hours. Polyadenylated [poly$(A)^+$] RNA was isolated by oligo(dT) cellulose chromatography. First strand cDNA synthesis was primed with an oligo(dT) linker-primer that contains a *Xho* I site and reverse transcribed using a RNase H^- reverse transcriptase in the presence of 5-methyl dCTP to hemimethylate the cDNA. Second strand cDNA was prepared

using RNase H and DNA polymerase I and the double-stranded DNA treated with Klenow before ligation to *Eco* RI adapters. The cDNA was ligated to *Eco* RI and *Xho* I restricted arms of the lambda phage Uni-ZAP XR vector. Phage were packaged and used to infect the *recA*$^-$ *E. coli* XL1-Blue MRF'. Approximately 3 x 10^5 recombinant plaques were transferred to Hybond N$^+$ membranes and screened with the genomic subclone pG1Ve. Eight cDNA clones were recovered and the pBluescript SK(-) phagemid with the cloned insert excised and recircularized.

Southern and transcription analyses. Genomic and cDNA clones linked to *Ve* were subcloned into the pBIN19 or pBI121 binary vectors for *Agrobacterium tumefaciens* mediated transformation of potato (19). Stable integration of the sequences between the T-DNA borders was confirmed by Southern analysis (20). Isolated genomic DNA was restricted, separated by gel electrophoresis, transferred to Hybond N$^+$ membranes (Amersham), and hybridized with pC1Ve as recommended by the membrane manufacturer. Expression of *Ve* in potato was determined by reverse transcription and amplification with the polymerase chain reaction (PCR). Total RNA was isolated from fresh plant material (TRIZOL, Life Technologies) and 2.5 µg of RNA treated with RNnase-free DNase I (Life Technologies) prior to cDNA synthesis with the ThermoScript RT-PCR System (Life Technologies). Reverse transcription was performed at 68°C (*Ve*1) or 58°C (*Ve*2) with 500 nM of the gene-specific primer 5'CTGGTTTCAACTCTGAAGTATC3' (*Ve*1) or 5'ATTTGCTGCCCCTACTATGTATCC3' (*Ve*2) complementary to 3' untranslated sequences. Subsequent PCR was performed with an annealing temperature of 68°C (*Ve*1) or 66°C (*Ve*2) in a 50 µl volume containing 10% of the cDNA reaction, 0.2 µM of the cDNA primer, and 5'TAACAGTCTTGTTGATCGTTTCCC3' (*Ve*1) or 5'TGAATTGTAAGTTGTTGGAGGTCC3' (*Ve*2) primers specific to 5' exon sequences.

Resistance complementation assays. Plants propagated in the greenhouse were inoculated with aggressive isolates of *V. albo-atrum* race 1 (18) or *Phytophthora infestans* US8 mating type A2 (21). Disease reactions were obtained by challenging a minimum of 10 plants from at least three independent lines of transgenic potato plants for each construct. Plants approximately 15 cm in

height were inoculated with *V. albo-atrum* by removing the lower roots before submerging the remaining roots for 10 min in a 5 x 10^7 conidia/mL suspension. Alternatively, leaves and stems were immersed in a 5 x 10^4 sporangia and zoospore suspension of *P. infestans*. Plants were rated three weeks postinoculation as either susceptible to the pathogen as indicated by chlorosis and necrosis of the leaves and stunting of the plant or as resistant if there were no disease symptoms and appearance was similar to the uninoculated plants.

Confined field trials of the Desiree plants containing the *Ve* genes were conducted in 1999, 2000, and 2001 at Outlook, SK, Vauxhall, AB and Winkler, MB.

Sequence analysis. Genomic DNA and cDNA sequences were determined with a Sequitherm Long Read Cycle Sequencing Kit (Epicentre Technologies) and an ABI 377 automated sequencer (PE Biosystems) using primers derived from the genomic sequences and the polylinker cloning site of the vectors. Various versions of the BLAST algorithm (22) were used to search DNA and protein databases for similarity. Motifs were identified with the PCGENE program (IntelliGenetics Inc.) version 6.8.

Positional cloning of *Ve* and genomic complementation in transgenic potato plants. To proceed with map-based cloning of *Ve*, we used the SCAR sequences as hybridization probes to identify λ clones that possessed contiguous, overlapping inserts of genomic DNA (Fig. 1) from resistant *L. esculentum* VFN8 germplasm. Identification of *Ve* involved *in vivo* functional complementation within the potato (*Solanum tuberosum* ssp. *tuberosum*) cultivar Désirée, which is highly susceptible to verticillium wilt. *In vivo* complementation and specificity was initially observed in potato plants transformed with the 4 kb genomic sequences of λ subclone pG2Ve and subsequently pG3Ve (Fig. 1). These plants exhibited delayed and reduced disease symptoms following inoculation with *V. albo-atrum* race 1 (Fig. 2A) but no non-specific resistance to *Phytophthora infestans* (Table 1). All untransformed plants and control plants transformed with the binary vector pBIN19 or pG1Ve displayed wilt, chlorosis, and necrosis within a few weeks of *V. albo-atrum* race 1 inoculation. All transformed plants showed increased vigour in the field as

compared to the negative controls (Table 2). These genes have been inserted into commercial varieties designated by the Saskatchewan potato producers (Fig. 2B).

Identification of expressed sequences and cDNA complementation in transgenic potato plants. To identify expressed sequences and the *Ve* locus, the λ subclones were sequenced and pG1Ve was used to probe a *L. esculentum* cDNA library of the verticillium wilt resistant tomato variety Craigella. Genomic sequences confirmed that pG1Ve possessed the SCAR sequence linked to the resistant *Ve* allele and revealed inverted terminal open reading frames (ORFs) in pG1Ve homologous to the N-terminal domain of plant and animal receptors that possess leucine-rich repeats. The cDNA clones, pC1Ve and pC2Ve, corresponding to the ORF sequences detected in the genomic subclone pG1Ve were isolated and designated *Ve*1 and *Ve*2 (Fig. 1). To confirm complementation observed with the genomic clones, the cDNA of pC1Ve and pC2Ve was cloned into the binary vector pBI121 in a sense orientation under transcriptional control of the cauliflower mosaic virus (CaMV) 35S promoter for plant transformation. All plants expressing pC1Ve and pC2Ve cDNA were resistant to *V. albo-atrum* race 1 but not *P. infestans*, whereas untransformed germplasm and plants transformed with the vector alone were susceptible to both pathogens (Table 1).

Deduced primary structure of *Ve*1 and *Ve*2. Sequence analysis of the isolated cDNA and corresponding genomic clones did not detect any introns within the *Ve* ORFs. However, an amino acid homology of 84% and several structural domains were observed within the deduced *Ve* proteins (Fig. 3). A hydrophobic N-terminus in the *Ve* proteins (domain A), indicative of a signal peptide that may target the protein to the cytoplasmic membrane (23), contains a leucine zipper-like motif with four contiguous amphipathic heptad repeats in *Ve*1. Domain A precedes a leucine-rich repeat (LRR) with 38 imperfect copies of a 24 amino acid consensus XXIXNLXXLXXLXLSXNXLSGXIP (domain B) that is often associated with protein-protein interactions and ligand binding. The presence of a glycine within the consensus sequence is consistent with that of extracytoplasmic proteins, a location that would facilitate the recognition of an extracellular pathogen ligand (14, 15). Within the predicted LRR region, 28 or 35

sequences matching the N-glycosylation consensus sequence NX(S/T) were observed in *Ve*1 and *Ve*2, respectively (Fig. 3).

As frequently observed with membrane-spanning proteins, a hydrophobic sequence with a predicted α helix secondary structure (domain D) is flanked by a negatively charged extracytoplasmic domain C and a positively charged cytoplasmic domain E. Each cytoplasmic domain possesses the dileucine E/DXXXLϕ or tyrosine YXXϕ signal sequences, where ϕ is an amino acid with a hydrophobic side chain, that stimulates receptor-mediated endocytosis and degradation of mammalian cell surface receptors (24). In *Ve*2, the C-terminus also contains a PEST-like sequence found in proteins with cytoplasmic half-lives of only a few hours (25) and concludes with the residues KKF, similar to the KKX motif that signals endoplasmic reticulum retention in mammalian and plant cells (26, 27).

Verticillium wilt resistance has been incorporated into most commercial tomato varieties and has proven to be very durable. We report in this study the positional cloning of the verticillium wilt *Ve* resistance genes from tomato. Identification of two closely linked inverted genes independently conferring resistance to the same pathogen was unexpected. The only other example of two closely linked functional disease resistance genes is the *Cf*2 locus that consists of direct repeats differing by only three nucleotides (28). Like *Cf*2, the *Ve* genes likely resulted from relatively recent gene duplication and homologous recombination events that are believed to contribute to R gene evolution. It is possible that the *Ve* receptors recognize different ligands which would require a pathogen to possess at least two virulence products before inciting disease.

Homology to the *Ve* genes was observed in several plant proteins with LRRs including disease resistance genes that probably produce cytoplasmic proteins (6-13) and the *Xa* and *Cf* genes (14, 15) reported to encode proteins with an extracytoplasmic domain that interacts with an extracellular ligand. Unlike *Xa*21, the *Ve* genes do not include a protein kinase and are unique being the only disease resistant receptors containing endocytosis-like signals and leucine zipper

or PEST sequences. Leucine zippers have been reported in the cytoplasmic class of *Arabidopsis* resistance genes *RPS*2 and *RPM*1 for *P. syringae* and can facilitate dimerization of proteins through the formation of coiled-coil structures (29). PEST sequences are often involved in ubiquitinization, internalization, and degradation of proteins (30). Discovery of a leucine zipper, PEST, and endocytosis signals in the *Ve* receptors expands the available motifs and complexity of plant cell receptors.

All eukaryotic cells exhibit receptor-mediated endocytosis as a mechanism to communicate or respond to external stimuli. In mammalian cells, ligand-dependent (e.g. insulin and cytokine CD4 receptors) or constitutive (e.g. transferrin and LDL receptors) endocytosis is stimulated by tyrosine or dileucine motifs located within the cytoplasmic C-terminus, concentrating cell surface receptors into clathrin-coated pits that are internalized and degraded in the lysosome (31). In plant cells, clathrin-coated pits and other indirect evidence of receptor-mediated endocytosis has been observed (32). Identification of the EKCLLW and YCVF sequences in the short cytoplasmic domains of two homologous but independent protein sequences indicates that plant and mammalian cell-surface receptors may share similar endocytosis signals. In *Ve*, receptor-mediated endocytosis could provide a mechanism through which cells selectively capture ligands and remove signaling receptors from their surface, thereby actively responding to changing disease pressures.

Cytoplasmic signaling by *Ve* may be analogous to that of the erythropoietin cytokine receptor. Preformed dimers on the cell surface facilitate transmission of a ligand-induced conformational change from the extracytoplasmic to the cytoplasmic domain and subsequent signal transduction (33). The cytoplasmic domain interacts with kinases that link ligand binding to tyrosine phosphorylation of various signaling proteins and transcription activation factors. A similar model has been proposed for the kinase encoded by the *Pto* resistance gene that lacks a receptor domain (6) and *Cf* extracytoplasmic receptors that lack a kinase (14, 28). Alternatively, receptor-mediated endocytosis may allow the extracellular domains and ligands of the *Ve* proteins to directly stimulate signal transduction.

Resistance to different pathogen species is contrary to the traditional view of a highly specific interaction with race-defining *R* genes. Our results demonstrate, that while the tomato *Ve* genes have the specificity to distinguish races 1 and 2 of *Verticillium dahliae,* the genes also possess the capacity to recognize another *Verticillium* species in a different host. This pleiotropic resistance resembles that observed with the *Mi* gene that confers resistance to nematodes and aphids (34, 35) and shares the ability of R genes to retain biological activity in other plant genera (8, 36). Several *Verticillium* species infect many agricultural plants and this pleiotropic, host-independent complementation should be of considerable value.

Patents have been applied for in the US, Canada and several other potato producing countries (Appendix 1). Sequences have been assigned GenBank accession numbers AF232366 (Ve1cDNA), AF272367 (Ve1 genomic), AF272368 (Ve2 cDNA), and AF272369 (Ve2 genomic).

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Fig 1. Schematic genetic and physical representation of *L. esculentum* linkage group (A) bordering the *Ve* gene. Analysis of populations segregating for *Ve* identified closely linked codominant RAPDs and allele-specific SCARs that map to the region of RFLP GP39. Identification of contiguous λ genomic clones (B) facilitated the subcloning of genomic DNA (C) containing the *Ve* locus. Vertical lines indicate location of the AUG initiation codon in the subgenomic clones. Expressed sequences were cloned into λ and arrowheads depict the direction of transcription for the cDNAs (D) identified using the genomic clone pG1Ve. Potato plants transformed with the genomic subclones pG2Ve and pG3Ve or cDNA pC1Ve and pC2Ve exhibited *in vivo* complementation and resistance (R) when challenged with *Verticillium albo-atrum* race 1. Potato plants transformed with the genomic pG1Ve and the binary vectors pBIN19 and pBI121 were susceptible (S) to infection.

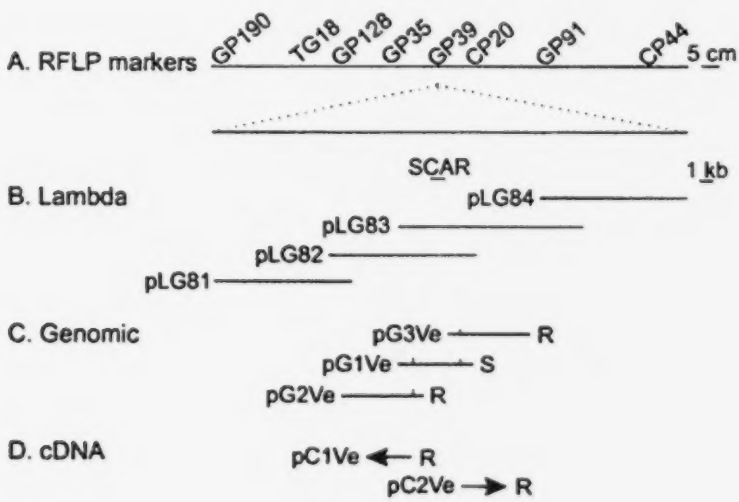
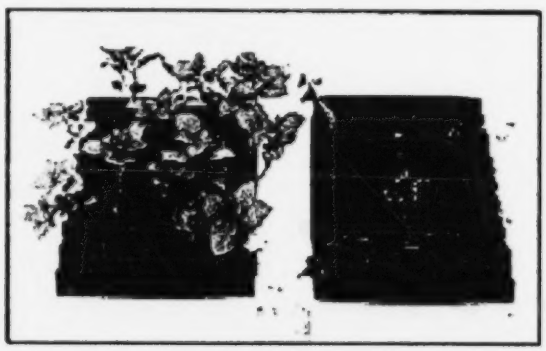


Fig 2. A. Genetic complementation in potato plants transformed with *Ve*. Disease symptoms were recorded three weeks after plants containing pG2Ve::pBIN19 (left) (resistant) or pBIN19 (right) (susceptible)were inoculated with *Verticillium albo-atrum* race 1. Similar disease resistance was observed in plants transformed with pG3Ve::pBIN19, pC1Ve::pBI121, and pC2Ve::pBI121. **B.** Field trials in Outlook, SK.

A.



B.

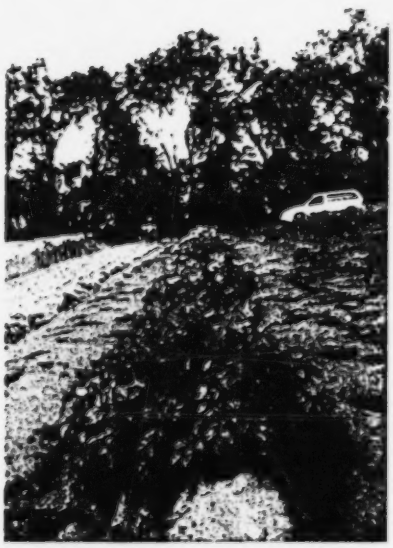


Fig 3. Primary structure of the *Ve*1 (A) and *Ve*2 (B) proteins deduced from cDNA sequence. The polypeptides have been divided into domains **A** through **G** as described in the text A dashed line occurs above the putative N-terminal leucine zipper in domain **A** of *Ve*1 and below the endocytosis signals in domain **E** of *Ve*1 and domain **G** of *Ve*2. Highlighted are the hydrophobic amino acids (purple) of the putative signal peptide domain **A** and membrane-associated domain **D**; conserved L/I (red), G (green), and potential N-glycosylation sites (blue) within the leucine-rich repeat (LRR) domain **B**; neutral and acidic amino acids (grey) of domain **C**; neutral and basic amino acids (yellow) of domain **E**. The PEST sequence of *Ve*2 is shown in domain **F**.

A.

B.

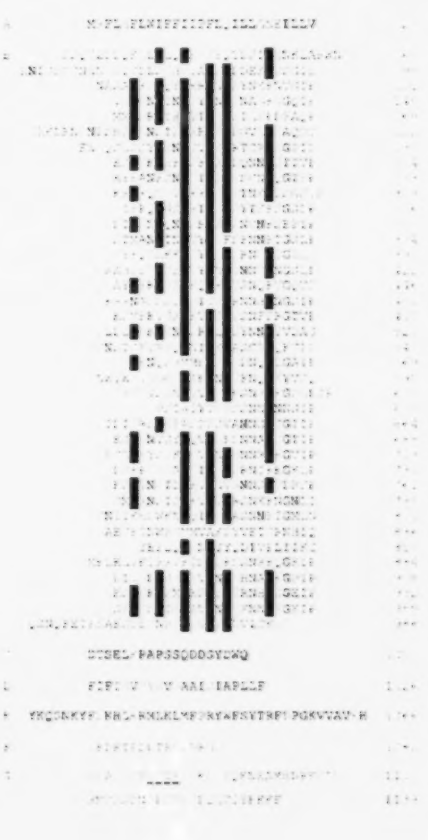


Table 1. Disease incidence in potato and tomato genomes challenged with aggressive isolates of *Verticillium albo-atrum* race 1 and *Phytophthora infestans* A2 US8. Plants were rated three weeks postinoculation as resistant (R = no disease symptoms) or susceptible (S = advanced necrosis). Complementation was observed only in plants challenged with *Verticillium albo-atrum* race 1 expressing full-length genomic or cDNA of *Ve*1 (pG2Ve and pC1Ve) or *Ve*2 (pG3Ve and pC2Ve).

Line*	*Verticillium albo-atrum* race 1		*Phytophthora infestans* A2 US8	
Potato				
	R	S	R	S
pG1Ve	0	31	0	8
pG2Ve	48	0	0	8
pG3Ve	83	0	0	8
pC1Ve	30	0	0	8
pC2Ve	78	0	0	8
pBIN	0	48	0	8
pBI122	0	48	0	8
Desiree	0	56	0	8
Tomato				
Ailsa Craig	0	32	0	32
Craigella	32	0	0	32
Tomato Total	32	32	0	64

* Transformants pG1Ve, pG2Ve, and pG3Ve contain genomic DNA with the intergenic region and N-terminus of *Ve*1 and *Ve*2, a full-length clone of *Ve*1, or a full-length clone of *Ve*2, respectively. Plants transformed with pC1Ve (*Ve*1) and pC2Ve (*Ve*2) express full-length cDNA clones under the transcriptional control of the CaMV 35S promoter.

Table 2. Yields of plants in Outlook, Saskatchewan field trials.

Tuber Yield Data 2000						
Clone	Large (>80 mm)		Medium (45-80 mm)		Small (<45 mm)	
	Number	Weight (kg)	Number	Weight (kg)	Number	Weight (kg)
Ve1G	0	0	54	13.09	75	5.42
Ve2G	0	0	47	10.51	107	8.14
Ve1C	0	0	42	8.69	88	7.9
Control	0	0	38	8.04	152	10.87

Tuber Yield Data 2001	
Clone	Weight (kg)
Ve1G	45.67
Ve2G	30.37
Ve1C	45.09
Control	41.46

Objective A2 - Late Blight Resistance

A strategy similar to that used in isolating the *Ve* genes has been applied to isolating the 12 potato resistance genes (R1 through R12) that confer complete race-specific resistance to the fungus *Phytophthora infestans* (Fig. 1). R1 has been mapped previously to potato chromosome V and R3 to chromosome XI. Markers linked with R3 were found also to be linked with R6 and R7. The resistance alleles R6 and R7 mapped to a similar distal position on chromosome XI as the R3 allele. Isolation of the R genes allows the shuffling of resistance on demand to reduce or eliminate the need to spray for late blight.

Tissue culture plants of the 11 R-gene differentials for *Phytophthora infestans* (R1 to R11) were grown in growth rooms until they started to bloom. Crosses were made carefully so as to avoid unintended pollination. The crosses were labelled and bagged for collection of any resulting seed balls. By early January, 2001, all the seeds had been removed from the seed balls, rinsed, dried and stored in envelopes.

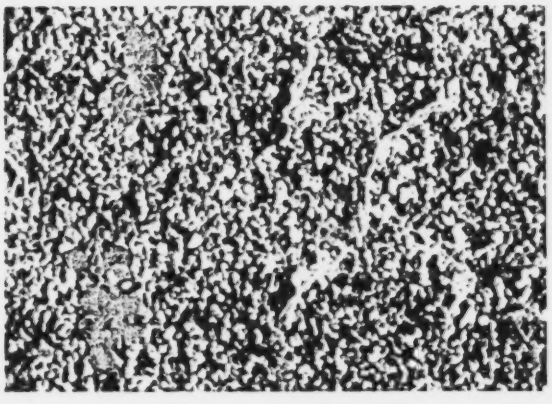
The following 23 crosses produced seed (listed with the female parent first):

R1 with R2, R6, and R8
R2 with R6, R8, and R11
R4 with R2, R6, and R8
R5 with R8
R6 with R8
R7 with R2, R6, and R8
R8 with R2
R9 with R2, R6, R8, and R11
R11 with R2, R4, R6, and R8.

In addition, the R2, R8, and R11 lines self-pollinated. The seed was stored at 3-4C. .

Seeds from the cross, R11 x R6, were allowed to germinate, and transplanted to 4" square pots. This yielded 81 plants to screen for the presence of R-genes 6 and 11. Screening was carried out on 10 leaf discs cut from the 3rd to 7th leaves on a shoot using 0a #10 cork borer. *Phytophthora infestans* isolate BE1 (A1 or A2) carries a pathogenicity profile of A 2, 4, 6 and 9 was used to inoculate the discs. Russet Burbank, Shepody, Stirling, and R6 and R11 plants were included in each trial as controls. As expected, R6 was inherited as a single dominant gene and a 1:1 segregation ratio was observed. A putative *P. infestans* resistance gene has been isolated via chromosome walking using closely linked DNA markers. This work is expected to continue if funding allows.

Fig 1. Genetic complementation in potato plants possessing a race-specific late blight R gene. Disease symptoms were recorded three weeks after susceptible (four plants on right) or resistant (two plants on left) were inoculated with an aggressive isolate of *Phytophthora infestans* US8.



Objective A3 - Enhancing General Resistance to Fungi

Three different constructs of the plant osmotin gene that hydrolyzes fungal cell walls have been prepared to overexpress the enzyme, alter cellular deposition, or eliminate expression of the gene. Potato plants have been screened and selected for each construct based on osmotin expression levels. Greenhouse screening trials have shown little alteration in resistance to *Pythium, Phytophthora* and *Fusarium* species as a function of over-expression of the osmotin gene. Although others have published results indicating that osmotin provides some resistance against these pathogens, our results show that this strategy needs to be improved to be of value to producers. These may include higher expression levels or cellular deposition that is more effective.

B. Virus Resistance

Objective B1 - Virus Movement

A new strategy has emerged that provides resistance to all potato viruses using a single viral gene. Harvest yields for the potato crop are severely affected by viral infections by the luteovirus PLRV (potato leafroll virus), the potexvirus PVX (potato virus X), and the potyvirus PVY (potato virus Y) (1). Classical strategies of breeding for resistance (e.g., to PLRV infections) are impeded by the absence of genes that would confer localized hypersensitivity. Transfer of resistance genes into high-yielding potato cultivars is a time-consuming process due to the tetraploid potato genome. Unwanted effects like a reduction in yield and fertility are often observed following transfer of the dominant PVY resistance gene Ry from *Solanum stoloniferum* (1). Alternative, nonconventional strategies for the production of virus-resistant crop plants have exploited the gene transfer technology for molecular resistance breeding (2). Recent experiments have demonstrated that the expression of a defective movement protein (MP) can confer broad-range resistance to infection by several unrelated viruses (3). Unlike the coat protein strategy, defective movement protein is licensable and would facilitate commercialization of virus resistant potatoes, achieved through introduction of defective virus movement proteins.

MP-mediated resistance strategy was used to produce potato lines capable of resisting infection by the viruses PVY, PLRV, or PVX. The strategy makes use of a gene (ORF4), which encodes a 17-kD protein (pr17; Fig. 1A), of the phloem-limited luteovirus PLRV. This PLRV gene is nested within the sequence for the coat protein gene, but uses a different reading frame. Biochemical characterization of pr17 has identified protein domains with specific activities: (1) the C-proximal basic domain binds to single-stranded nucleic acids in a sequence-unspecific manner (4) while (2) the N-proximal acidic domain contains an amphipathic alpha-helix that mediates protein-protein interactions in the formation of oligomers (5). Pr17 copurifies with membrane fractions, and it localizes in both PLRV-infected and transgenic plants to the plasmodesmata (5,6). On the basis of these characteristics and by analogy to the movement protein of barley yellow dwarf luteovirus (7), we concluded that pr17 represents a phloem-specific luteoviral PLRV movement protein (5,6,8). We observed that expression of mutant versions of PLRV pr17 in transgenic potato plants and on their potential in inducing broad-range protection against infection by the viruses PVY, PLRV, and PVX.

Results

Expression of pr17 wild-type and variant proteins in transgenic potato plants. Mutant PLRV pr17 movement proteins that retain their capacity for oligomerization via the N-terminus and nucleic acid binding by the C-terminus, would supposedly form heteromultimers with wild-type pr17 of an infecting virus, thereby acting as dominant negative mutants. Previous experiments have shown that deletion derivatives of pr17 may lose their capacity for multimerization (5), therefore pr17 mutant alleles were constructed with short hydrophilic extensions to the N-terminus or to the C-terminus. In the allele N (Fig. 1B) the resulting pr17 variant (pr17-N) contains a 2-kD peptide N-terminal extension. The allele NP for the pr17-NP protein was constructed from allele N by the conversion of two amino acids within the amphipathic alpha-helix into structure-breaking proline residues. This modification of the oligomerization domain was previously shown to abolish the capacity for multimer formation (5). According to the putative protection mechanism described above, pr17-NP represented the negative control. A 3' terminal variant of the pr17 gene was produced by fusing part

of the GUS reporter gene to the 3' end, which resulted in a 5-kD hydrophilic extension of the pr17 C-terminus (pr17-C). The proteins encoded by alleles N and C in fact retained their capacity to form dimers with wild-type pr17 as tested in vitro (Fig. 2). The modified pr17 genes as well as the pr17 wild-type gene (WT) were put under the transcriptional control of the CaMV 35S promoter pnd terminator and used for Agrobacterium-mediated transformation of leaf discs prepared from the potato cultivar Desiree.

Transgenic calli were selected on kanamycin-containing medium, and for each construct 10 independent transgenic lines were regenerated. For each construct, two of these lines were selected on the basis of high-level gene expression. Plants expressing the pr17-NP variant protein, which is not able to multimerize, did not accumulate detectable amounts of protein (Fig. 3B), although transcript levels of the transgene were comparable to those of the other transgenic plant lines (Fig. 3A). As all constructs contained an ATG start codon with optimal flanking sequences for translational initiation in plants (9), it was unlikely that the differences in protein accumulation were due to different translation initiation efficiencies. The transgenic plants were further characterized with respect to the subcellular localization of the various mutant pr17 proteins. Cell fractionation studies (data not shown) showed that the modified proteins, prl7-N and -C, were associated with the membrane-rich fractions to which the wild-type pr17 is localized (5).

Resistance tests with the luteovirus PLRV, the potexvirus PVX, and the potyvirus PVY. The two highest expressing lines for each of the transgenes pr17-WT (4, 6), -N (10, 11) -NP (11, 14) and -C (3, 11) were vegetatively propagated together with the nontransgenic control line D (untransformed Desiree plants) and a transgenic control line DB that contains the T-DNA of the binary vector pBIN19. For each line 15 plants were used in the resistance trials. For the mechanically nontransmissible luteovirus PLRV, infection experiments were performed by grafting scions from a systemically PLRV-infected potato line onto the transgenic plants and the non-transgenic Desiree control plants. To evaluate virus protection during secondary infection, tubers were harvested from these plants and regrown in soil after a storage period of 4 to 8 weeks at 4 C. Seven weeks after shoot emergence the virus antigen was determined separately for each plant and

analyzed in two independent experiments (Fig. 4A, B). In both resistance trials, all transgenic plant lines, with the exception of line NP- II, showed a significant reduction of the virus antigen compared to plants transformed with just the pBIN19 binary vector or the non-transgenic controls (Fig. 4A). Reduction of viral antigen in the pr17-transgenic lines, as compared to the non-transgenic control line, varied from 29% to 72%. No statistically significant differences in virus resistance were detected between plant lines carrying the various constructs Therefore, it was not possible to classify the different transgenic lines into distinct resistance groups.

Resistance to infection by PVX was tested in the same transgenic Desiree lines because this cultivar is susceptible to PVX infection. For each line, 15 plants were mechanically inoculated, and 3 weeks after inoculation leaflets from the first two leaves above the inoculation sites were harvested for the determination of PVX antigen levels (Fig. 4C). After 6 weeks, leaflets from the third and fourth leaf above the inoculation sites were analyzed for PVX antigen (Fig. 4D). Transformed lines expressing either the transgenes for WT pr17 (lines 4 and 6) while the NP lines (11, 14) did not show any significant reduction of virus antigen. On the contrary, only those lines accumulating modified versions of pr17 resulted in a significant reduction of virus antigen, with lines carrying the N-transgene performing significantly better than those with the C-transgene. In comparison to the nontransgenic control plants, virus antigen in N-transgenic lines was reduced by 55% to 91%.

In order to test for resistance to PVY, the pr17 mutant allele N (N- transgene) that performed best in the PVX resistance test was used to transform the PVY-susceptible potato cultivar Linda (the potato cultivar Desiree displays field resistance to PVY infection). Twenty independent transgenic lines were produced and analyzed for gene expression by Western blot analysis. Six lines with high pr17-N accumulation (Fig. 5A) were used in the resistance test, as their steady-state levels of pr17-N were even higher than those observed for the N-transgenic Desiree lines (data not shown). Fifteen plants for each line, together with nontransgenic plants of cultivar Linda (L) and plants transgenic for the T-DNA of the binary vector pBINI9 (LB), were mechanically inoculated with the common strain of PVY (PVYo). Two weeks post infection (p.i.) material from the first and second leaf above the inoculated leaf were sampled and the PVY antigen level determined (Fig. 5B). A second

determination for the establishment of primary infection was done 4 weeks p.i. in the third and fourth leaf above the inoculation site (Fig. 5C). At this stage, the inoculated leaves in the control plants as well as the leaves below the inoculation point showed severe necrosis. In pr17-N transgenic plants these symptoms were confined to the inoculated parts of the leaf. With the exception of lines 5 and 8, for which 4 weeks after inoculation the reduction of virus antigen was approximately 80%, the other four N-transgenic lines (L2, L4, L6, and L7) showed a reduction in virus antigen of more than 90% compared to the nontransgenic control (Fig. 5C). As the common 0 strain of PVY causes severe necrosis resulting in loss of leaves, ELISA measurements were not done at later time points.

Pathogenesis-related genes are not expressed in uninfected transgenic plants. The results suggested that protection against infection by the viruses PVX and PVY was achieved by the accumulation of N- and C-terminally extended versions of PLRV pr17. However, high-level accumulation of these proteins could generate a state of stress in the plant. In fact, sucrose and starch metabolism was altered in the transgenic plants depending on the genotype and the transgene (unpublished data). This stress state could mimic an infection situation and possibly result in the expression of pathogenesis-related (PR) genes that are expressed upon pathogen infection and thought to contribute to an active defense mechanism of the plant referred to as "induced resistance". Several different PR genes are induced upon virus infection and are classified into five groups; for two of these groups functions have been attributed to the corresponding proteins (group 2: glucanases; group 3: chitinases) (10). In order to exclude the possibility of PR-mediated resistance, the transgenic Desiree lines with the highest resistance to PLRV and PVX were tested by Western blot analysis with antisera directed against potato glucanases and chitinases. Under the conditions employed, the anti-glucanase sera detected a 36-kD protein and the anti-chitinase sera detected a 33-kD protein only in PVX- infected potato plants, but not in the uninfected transgenic lines nor in the uninfected nontransgenic lines (data not shown).

Discussion

These results demonstrate that the stable transformation of potato by a single construct induces

broad-spectrum resistance against the unrelated potato viruses PLRV, PVY, and PVX. This phenomenon of broad-spectrum resistance is based on the expression of a mutant allele of the PLRV gene ORF4, which encodes a 17-kD protein (prl7). As we have shown (4,5), this protein displays biochemical characteristics of a viral movement protein, it is subject to phosphorylation by membrane-bound kinase(s) (data not shown), and it is localized to plasmodesmata(6). As the transgenic plants did not express pathogenesis-related proteins like glucanases or chitinases, it is unlikely that the resistance described here is brought about by the activation of a general plant defense mechanism. Rather, the induced resistance results from expression of the introduced transgene. According to our interpretation of long-distance movement of plant viruses through the phloem tissue(11), pr17 mutant proteins localized to the plasmodesmata of the sieve element-companion cell complex should be capable of interfering with systemic spread of unrelated viruses by inhibiting cell-to-cell movement. A similar hypothesis for the possible mechanism of broad-range protection mediated by the expression of a defective MP was suggested recently in that defective MPs might preoccupy target sites in plasmodesmata and thereby interfere with the accumulation of wild-type protein.

Our results suggest that the observed broad-spectrum protection requires the accumulation of N- or C-terminally modified pr17 proteins. NP-transgenic plants that do not accumulate detectable amounts of mutant protein. The 17 WT-expressing plants did not exhibit resistance against infection by PVX. It is not surprising that multiplication of heterologous viruses is not enhanced in the pr17 WT-expressing plants. These viruses spread beyond the phloem tissue, but the movement protein of a phloem-limited luteovirus is supposedly less active in cells other than those of the phloem system. In fact, localization studies of WT pr17 in transgenic plants reveal that pr17 associates preferentially with plasmodesmata of the sieve cell-companion cell complex and displays a lower affinity to plasmodesmata connecting mesophyll cells (data now shown).

Whereas symptoms produced by PVX are difficult to determine, it is known that the 0 strain of PVY causes necrosis in sensitive lines, which enables an assessment of symptom development. As inferred from a comparison of the phenotype of PVY-infected transgenic and control potato plants,

symptom development was delayed not only in the systemic (Fig. 5A), but also in the inoculated leaves (Fig. 5C, D). Similarly, a reduced accumulation of heterologous virus was reported in the inoculated leaves of brome mosaic virus MP-transgenic plants(13).

Transgenic potato plants showed a reduction in PLRV titer for a transgene that was only expressed at the RNA level (NP), as well as for transgenes expressed also at the protein level (N, C, WT). Similar results on induced PLRV resistance by RNA expression of a virus-derived gene were obtained with transgenic potato plants expressing the PLRV coat protein (CP) gene(14). Barker et al.(15) reported that resistance to PLRV is related to the amount of PLRV CP RNA. Indeed, induced resistance to PLRV differs from that observed with other plant viruses in that it is conferred equally efficiently by both sense and antisense RNA expression of the CP gene (16). It was suggested that transgene transcripts interfere with virus replication by interacting with the opposite RNA strand. Alternatively these RNAs might compete for essential viral or host factors. As the PLRV pr17 gene is nested within the CP gene, the resistances described here might relate to the same RNA-mediated mechanisms. Such a resistance mechanism would explain why the PLRV virus titer is also reduced in plants that express wild-type pr17.

Conclusions

Movement protein can provide resistance to all the viruses that infect potato and would be available to SSPGA. Further work should be able to increase the levels of resistance.

Experimental protocol

Vector construction, dimerization experiments and potato transformation. Gene N encodes a pr17 variant (pr17-N) with the peptide MAELGSGSELHRGGGRSRTS fused to the N-terminus. This construct in which the multiple cloning site of the pBluescript vector is translationally fused to the pr17 gene has been previously described as p17/NIII (ref. 17). Gene N was further mutated in such a way that the two triplets for the amino acids A and E in the alpha-helix (corresponding to the multimerization domain of the protein (5)) were converted to proline codons to yield mutant protein

pr 17-NP (see NP in Fig. IB). Mutation protocols followed those described for the construction of plasmid mut2 (5). For the construction of gene C encoding a short C-terminal elongation of pr17 (pr17-C), a chimeric gene consisting of the pr17 gene and the GUS reporter gene(18) was used as a template for polymerase chain reactions with the primer combinations ATGTCAATGGTGGTGTACAAC (homologous to the 5' end of the pr17 gene) and TTCGCGCTGATACCAGACGTTG (complementary to the GUS gene). Sequences for all pr17 wild-type and mutant alleles were verified by sequence analysis. Dimerization experiments including construction of transcription vectors, in vitro transcription/translation and chemical cross-linking followed established protocols(5). For plant transformation, the corresponding segments were put under the control of the CaMV 35S promoter and terminator by subcloning into the pRT1O3 vector(19) and introduced into the binary vector pBIN19 (ref. 20). Transfer of plasmids to Agrobacterium tumefaciens and subsequent transformation of potato cultivars Desiree and Linda followed established protocols(5).

Northern blot analysis. Poly(A)+ RNA was isolated from potato leaf material(21), 4ug of each poly(A)+ RNA preparation were separated on 1.2% denaturing formaldehyde-containing agarose gels and blotted onto Hybond- N membrane. Northern blots were hybridized with a 32P-labelled single- stranded RNA probe transcribed from the PLRV antisense clone #5 (ref. 6) and processed as previously described(22).

Western blot analysis. Total protein was isolated from leaf material under denaturing conditions, separated by polyacrylamide gel electrophoresis (PAGE) on 12.5% sodium dodecyl sulfate-containing gels, electroblotted onto nitrocellulose membranes and processed for Western blot analysis(5). Membranes were incubated with a 1: 1,000 dilution of a rabbit anti-prl7 serum(5) or with 1:5,000 dilutions of antisera raised against the basic glucanase and basic chitinase, respectively, from potato. Filters were incubated with goat anti-rabbit IgG/horseradish peroxidase conjugate (Sigma, Diesenhofen, Germany) and with a peroxidase substrate using the Amersham chemiluminescence (ECL, Braunschweig, Germany) kit.

Resistance tests. Inoculations of plants with PLRV and the common 0 strain of PVY were carried out as previously described(23). PVX (strain X239) for infection was maintained on tobacco Samsun NN. Resistance to PVX was tested as previously described (24). Sap was prepared from

systemically infected tobacco plants by homogenization in 50 mM phosphate buffer, pH 7.0, and used as the source of inoculum. Before use, the sap was diluted in the same buffer to a final PVX concentration of 10 ug/ml. Three carborundum (400 mesh) dusted terminal leaflets of each plant received 25 uL of inoculum each. Leaflets of four different leaves for PLRV detection, and leaflets of two leaves for PVX and PVY detection, were pooled and stored at -20'C. Thawed leaves were homogenized in plastic bags. The extracts were diluted in cold sample buffer and analyzed using the immunoassay kits from Boehringer Mannheim (Germany) and following the instructions of the manufacturer. Processing of the probes was performed using a multi-washer (Tri-Continent Scientific), and ELISA readings were done with the integrated EIA management system (Labsystems). ELISA readings were normalized for the mean value obtained from uninfected, nontransgenic control plants. For statistical data analysis the MSTAT-C program (Michigan State University) was used.

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Figure 1. The PLRV genome and chimeric genes used for plant trans- formation. (A) Schematic representation of the PLRV RNA genome. The six major open reading frames (ORFs) are indicated by open boxes. The gene for the 17-kD movement protein (pr17) is represented by the shaded box. (B) Schematic representation of the various pr17 alleles. Gene WT encodes wild-type pr17. Gene N encodes a modified pr17 with a hydrophilic N-terminal extension. Gene NP is derived from gene N by site-directed mutagenesis resulting in the conversion of two amino acids of the amphipathic alpha-helix into structure-breaking proline residues. Gene C encodes a pr17 variant with a C-terminal extension produced by translational fusion to part of the GUS reporter gene. P: proline residue.

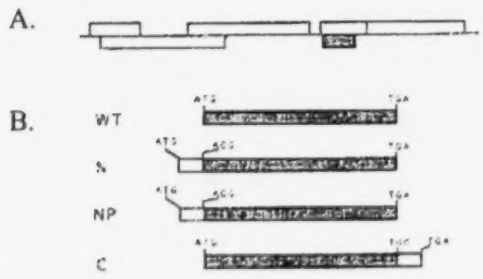


Figure 2. Analysis of homo- and heterodimerization. 35S-labeled pr17-WT, -N and -C proteins were synthesized by in vitro transcription/translation, and oligomers formed in solution were stabilized by covalent cross-linking in the presence of glutaraldehyde as described (5). (A) Dimerization of pr17-WT and pr17-N proteins. Incubation was done either with pr17-WT (lane 1) or pr17-N (lane 2) alone or with a mixture of both proteins (lane 3). The positions of pr17-WT homodimers (WT/WT), pr17-N homodimers (N/N) or heterodimers (WT/N) are indi- cated. (B) Dimerization of pr17-WT and pr17-C proteins. Incubation was done either with prl7-WT (lane 1) or prl7-C (lane 3) alone or with a mixture of both proteins (lane 2). The positions of pr17-WT homodimers (WT/WT), pr17-C homodimers (C/C), or heterodimers (WT/C) are indicated.

A. B.

Figure 3. Analysis of transgenic expression.(A)Detection of RNA transcripts by Northern blot analysis. The RNA samples were isolated from plants expressing the wild-type gene (WT-4 and WT-6), a pr17 gene encoding a protein with an N-terminal extension (N-10 and N-11), a pr17 gene for an N-terminally extended pr17 with two amino acids of the amphipathic alpha-helix exchanged by proline residues (NP-11 and NP-16), or a gene for a pr17 protein with a C-terminal hydrophilic elongation (C-3 and C-11). PLRV RNAs were detected by hybridization to a 32P-labelled PLRV RNA probe and autoradiography. Positions of RNA standards are indicated. (B) Detection of transgene-encoded proteins. Plants are the same as indicated in A. Total protein extracts were separated by SDS-PAGE, electroblotted onto membranes, and reacted with anti-pr17 sera. Immunoreactive proteins were detected by the ECL system. Molecular weights of prestained marker proteins are indicated.

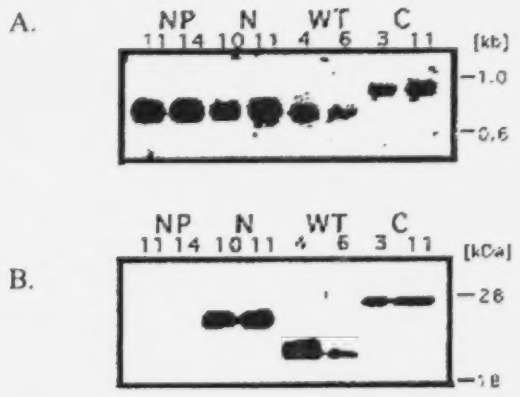
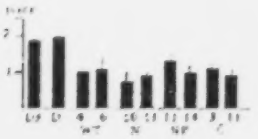


Figure 4. Accumulation of PLRV and PVX antigen in transgenic and control Desiree lines. (A. B) PLRV resistance test. Plants expressing the wild-type (WT) pr17 gene, mutant pr17 genes (N, NP, C), a trans- genic control line containing the T-DNA of the pBINI9 vector (DB), and nontransfornied control plants (D) were graft-inoculated with scions from PLRV-infected plants. Plants were grown to maturity, tubers were harvested and regrown for the estimation of secondary infection by determining ELISA values (A405). Each histogram represents the mean value obtained from 15 plants ± standard error (SE). Results in (A) and (B) were obtained from two independent experiments. (C, D) PVX resistance test. Plants of the transgenic lines described above were mechanically inoculated with PVX. Three weeks after inoculation leaf disks were harvested from the first and second leaves above the inoculated leaf (C). The leaves were pooled and ELISA values were determined by using anti-PVX sera. A second time-point was taken 6 weeks after inoculation (D) by determining viral antigen from the third and fourth leaves above the inoculated leaf, and ELISA values were determined. Each histogram represents the mean of fifteen plants ± SE.

A.



B.

C.

D.

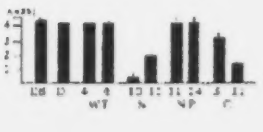
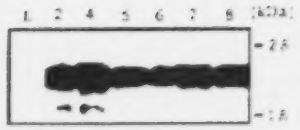
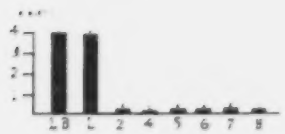


Figure 5. PVY resistance test of N-transgenic potato plants (cultivar Linda). (A) Western blot analysis of six N-transgenic potato lines (2, 4, 5, 6, 7, and 8) and one nontransgenic control line (L). Total protein extracts were subjected to Western blot analysis using anti-pr17 sera as described in the legend to Figure 3. (B, C) PVYo resistance test with the six N-transgenic potato lines (2, 4, 5, 6, 7 and 8), a transgenic control line containing the T-DNA of the vector pBIN19 (LB), and the nontransgenic control line (L). Fifteen plants per line were mechani- cally inoculated with the common 0 strain of PVY. Two weeks after inoculation, leaf disks were harvested from the first and second leaf above the inoculated leaf (B), and 4 weeks after inoculation from the third and fourth leaf (C). Each histogram represents the mean ELISA value from 15 plants +/- SE.

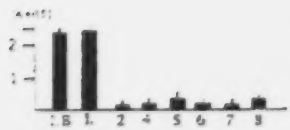
A.



B.



C.



Objective B2 - Interference withVirus Replication

Alternative strategies for virus resistance expected to deliver immunity are emerging as new virus genes are discovered. We have been very successful in this area. For example, mono- and polyclonal antibodies directed against different domains of the potato leafroll luteovirus (PLRV) P1 (ORF1) protein were applied to the analysis of P1 expression during PLRV replication in planta. Western analyses detected P1 and a protein of ~25 kDa (P1-C25) that accumulated to readily detectable amounts in PLRV-infected plants, but was not detected by in vitro cell-free translation of P1. P1-C25 represents the C-terminus of P1 and is a proteolytic cleavage product produced during P1 processing. On the basis of its biosynthesis, localization and biochemical properties, P1-C25 may facilitate the formation of P1/PLRV RNA complexes in which the spatial proximity allows for covalent bond formation between PLRV RNA and Vpg. Since the role of this protein is critical for viral replication, it represents a target that we can use to produce PLRV immunity.

Processing of polyprotein precursors into intermediates and mature proteins is a well-known phenomenon with animal and plant viruses. One example from the animal virus field is poliovirus: the positive-sense RNA genome of this picornavirus isfirst translated into a single polyprotein precursor that requires three different virus-coded proteinases for the maturation of viral proteins and completion of the infection cycle (1). During maturation of the polioviral primary translation product, several proteolytic intermediates containing the VPg and replication-associated protein sequences were identified by immunological techniques. One of these intermediates, which leads to the release of VPg and its covalent linkage to RNA, isthe P3-9 protein of 12 kDa that is abundantly associated with membrane structures of infected cells where replication of picornaviral RNA is known to occur (2).

In the plant virus field, potyviruses (3,4) are an example of positive-sense, single-stranded RNA viruses with a terminal RNA-linked VPg that share the translational strategy of poliovirus (processing of a primary polyprotein). In contrast, the expression of luteoviral genes occurs via subgenomic (sg)RNAs, but its VPg, as demonstrated recently for potato leafrollvirus (PLRV; 5), is

located within the C-proximal part of P1 (ORF1) from which it must be released through proteolytic cleavage. PLRV is a typical member of the luteovirus subgroup II (polerovirus), whose genome consists of a 5.8 kb single-stranded positive-sense genomic RNA (gRNA) with eight major open reading frames (ORFs) (6-9; Fig. 1A). The5[prime] gene cluster (ORFs 0-2) is highly divergent among luteoviruses and is separated from the 3[prime]-proximal group of relatively conserved genes (ORFs 3-7) by an untranslated region of ~200 nt located near the centre of the genome. The 3[prime] genes are expressed through the transcription of at least two sgRNAs, sgRNA1 (9,10) and sgRNA2 (11), and their translation involves non-canonical strategies (12) such as internal initiation and amber stop codon suppression. Both sgRNAs function as polycistronic mRNAs, and this also applies to PLRV gRNA: expression of the 5[prime] located genesP0 (ORF0) and P1 (ORF1) occurs from gRNA by initiation at their respective AUG codons. A -1 ribosomal frameshift within the 5[prime] end of the 582 nt overlap between P1 and P2 allows for the translation of P2 as a P1/P2 transframe protein (13,14). Functions attributed to these gene products are based on several lines of evidence, such as the development of disease symptoms in transgenic plants expressing P0 (15; D.Prüfer, unpublished), protein sequencing of the VPgN-terminus (5), or the presence of motifs characteristic of helicases (P1 and P2; 16), proteases (P1; 17), and RNA-dependent RNA polymerases (P2; 18).

As deduced from the putative functions of the various proteins, P1 apparently plays an important role in the replication of PLRV RNA. It is not only translated in at least two forms (P1, P1/P2 transframe protein), but it additionally must serve as a precursor for the generation of VPg (and possibly other proteolytic cleavage products of yet unknown function). In this study, we demonstrate by immunological analyses that P1 is in fact proteolytically processed and that one of the products (P1-C25)accumulates to readily detectable amounts in PLRV-infected plants. P1-C25 originates from the P1 C-terminus, it is localized in membranes and cytoplasmic fractions and exhibits nucleic acid-binding activity. A possible function of P1-C25 during VPg maturation will be discussed.

MATERIALS AND METHODS

Preparation of monoclonal antibodies (mAbs)

Immunization, myeloma cells and culture media. GST fusion proteins were prepared by cloning deletion derivatives of theORF1 coding sequence into the SmaI restriction site of the pGEX vector (19) as described in Figure 1B and purified from bacterial extracts by affinity chromatography on glutathione-Sepharose columns (Pharmacia). For the initial immunization,100 µg of GST fusion proteins were emulsified 1:1 (v/v) with complete Freund's adjuvants (Gibco BRL, Eggenstein, Germany). One half each of this emulsion was applied to Balb/c mice by intraperitoneal and intramuscular injection, respectively. At 3 and 6 weeks after the first immunization, booster immunizations were administered using incomplete Freund's adjuvants. Three days before removing the spleen for cell fusion, an intravenous boost was given with 50 µgGST-ORF1 fusion proteins in 50 µl of physiological saline. The serum titer was determined by enzyme-linked immunosorbent assays (ELISA; 20) in microtitre plates (Dynatech M 129B) as described by Clark and Adams (21) using affinity-purified alkaline phosphatase (AP)-conjugated goat anti-mouse antibody (Dianova, Hamburg, Germany) and ap-nitrophenylphosphate substrate (PNPP; Sigma, Deisenhofen, Germany). Only mice showing a GST-ORF1 fusion protein-specific antibody titer higher than 1:250 000 were used for the generation of hybridoma lines.

P3-X63-Ag8.653 myeloma cells (22) were grown in RPMI 1640 (Cytogene, Berlin, Germany) containing 15% fetal calf serum (Cytogene), 25 mM NaHC03, 1 mM l-glutamine, 50 µM 2-mercaptoethanol, 24 mM sodium bicarbonate, 50 IU penicillin and 50 µg streptomycin/ml (Gibco BRL). For selection of hybridoma cells, 100 µM hypoxanthine, 10 µMaminopterine and 16 µM thymidine (Sigma) were added to complete RPMI medium (HAT medium). After two limiting dilution cloning steps in 96-well microculture plates (Falcon), the cells were grown in suspension mass cultures at 37°C in a humidified incubator at 5% CO2.

Production and purification of mAbs. The fusion of myeloma and spleen cells was carried out

according to Westerwoudt(23). For in vitro production of mAbs, hybridoma cells were cultured in ten 250 ml tissue culture flasks each. The presence of ORF1-specific mAbs was determined by ELISA, and affinity-purified GST was used to identify and eliminate GST-specific mAbs during screening. Determination of mAb reactivity, affinity, isotypes and specificity was performed asdescribed (24). MAbs from hybridoma culture supernatants were concentrated by affinity chromatography on Prosep-A HC(Bioprocessing, Consett, UK). The purity of the mAb preparation was determined by SDS-PAGE (25).

Polyclonal antiserum. Polyclonal antiserum was prepared by emulsifying 1:1 (v:v) pP1-2 fusion protein with complete Freund's adjuvant and introduced into chickens by intramuscular injection. At 3 and 6 weeks after the primary immunization, booster injections were administered using incomplete Freund's adjuvant. Serum was extracted from eggs and titred as described for mAbs used in this study.

Immunological examination of PLRV P1 and proteolytic products in planta

Total protein was isolated under denaturing conditions from the leaves of healthy and systemically PLRV-infected *Solanum uberosum* and *Physalis floridana* plants according to Baunoch et al. (26). Aliquots of 10 µg were separated by 10%SDS-PAGE and electroblotted to nitrocellulose membranes as described by Niesbach-Klösgen et al. (27). Membranes were blocked overnight at 4°C in 1× phosphate buffered saline (PBS; 80 mM Na2HPO4, 20 mM NaH2PO4, 100 mM NaCl, pH7.5) containing 5% skimmed milk powder and 0.5% Tween 20. Immunodetection experiments were carried out according to established protocols (27).

To study the association of P1-C25 with subcellular structures, plant extracts were isolated under non-denaturating conditions and fractionated on discontinuous sucrose gradients as described by Niesbach-Klösgen et al. (27).

Purification and examination of PLRV

Isolation of PLRV virus particles from *S.tuberosum* was performed as described by D'Arcy et al. (28) with the following modifications. Cell walls were digested with 1% (w/v) cellulase (Sigma) and 8% (w/v) macerozyme (Serva) for 12 h priorto adding 2-mercapto-ethanol to a final concentration of 100 mM. Virus collected from rate zonal centrifugation on sucrosegradients was further purified by CsSO4 density gradient centrifugation and quantified by absorption at 260 nm and ELISA assay. Aliquots of 1 μg of purified PLRV and phenol/chloroform extracted viral RNA were treated with ribonuclease A and separated on 18% SDS-polyacrylamide gels.

Nucleic acid-binding assays

For in vitro nucleic acid-binding studies, bacterial extracts expressing the P1 (ORF1) deletion derivatives described inFigure 1B were separated by PAGE and blotted onto nitrocellulose membranes. Filter binding experiments were performed as described by Gramstat et al. (29) using a 32P-labelled PLRV probe obtained by in vitro transcription (30).

RESULTS AND DISCUSSION

Immunological analysis of the PLRV P1 protein in planta

A set of mono- and polyclonal antibodies directed against different domains of PLRV P1 (Fig. 1B) were raised and their activity characterized by ELISA and western blot analysis. Generation of antibodies against pP1-4 has not been successful to date, while mono- (pP1-1, pP1-2, pP1-3) and polyclonal (pP1-2*) antibodies showed a strong and significant reactivity to the corresponding antigens.

The results of immunodetection experiments with protein extracts from healthy and PLRV-infected *P. floridana* plants are shown in Figure 2. Only mAbs directed to the C-terminus of P1 (pP1-1,

pP1-3; lanes b and d) detected two immunoreactive proteins that were absent in the healthy control (lane a). The larger protein corresponded in size to P1, while the smaller product displayed a molecular weight of ~25 kDa (P1-C25). MAbs raised against epitopes within the central domain of P1(pP1-2) and reacting with the bacterially expressed P1 fusion proteins, were unable to detect the denatured P1 and P1-C25proteins (Fig. 2, lane c) from PLRV-infected plants. To further verify the in planta presence of P1 and define the location of the P1-C25 N-terminus, a polyclonal antiserum was prepared against pP1-2. As shown in Figure 2 (lanes e and f) the polyclonal antibody detected P1, but not P1-C25 confirming the origin of P1-C25 from the ORF1 C-terminus. In vitro cell-free translation of PLRV gRNA resulted in P1 as the only immunoreactive product (L. Kawchuk, unpublished), and additional host and/or viral factors may be required for P1 processing.

By direct VPg sequencing, van der Wilk and co-workers (5) localized the PLRV VPg within the P1 coding sequence and postulated its release by P1 proteolytic cleavage. As deduced from the apparent molecular weight of P1-C25, the N-terminiof P1-C25 and the VPg are located at approximately comparable positions within the P1 protein, indicating that P1-C25 may function as a possible precursor during VPg maturation or represent a product following VPg cleavage. The pP1-2 fusion protein (Fig. 1B) contains the entire VPg sequence as determined by van der Wilk et al. (5), but polyclonal antibodies directed against pP1-2 did not react with P1-C25, suggesting that VPg is either not present on P1-C25 or that it is not an efficient epitope in the fusion protein pP1-2. Only the availability of antibodies directed against the sequenced PLRV Vpg will allow us to distinguish between these two possibilities. With poliovirus, the VPg is stable in the infected cell and cell extracts, as long as it is part of the P3-9 precursor (2), whereas it is rapidly degraded in its free form. Therefore, it has been suggested that P3-9 may protect the VPg from cellular proteases through protein folding (31,32), and P1-C25 may serve the same purpose for PLRV VPg.

Immunological analysis of the PLRV P1 proteins in subcellular fractions

In addition to the mature poliovirus VPg-either bound to virion RNA or in its free form (33)-six precursor proteins containing the VPg amino acid sequence were detected in poliovirus-infected cells

(34). Only one of these, a 12 kDa protein, was found in membrane structures of infected cells where picornaviral replication is known to occur (2). Thus in an effort to study the subcellular localisation of PLRV P1 and P1-C25, immunological studies were performed with isolated fractions of nucleus/chloroplast, membranes/mitochondria, cytoplasmic proteins and cell wall proteins. As depicted in Figure 3, the P1-C25 protein was detected in the P30 (membrane), S30 (cytoplasmatic) and CW (cell wall) fractions. In addition, minor immunoreactive proteins of ~18 (P30) and ~6 kDa (S30) were detected which were also present in total protein extracts from PLRV-infected plants after incubation for 30 min at room temperature prior to loading on a gel. The high amounts of P1-C25 associated with membrane structures is surprising in view of the highly hydrophilic character of theP1-C25 protein. In contrast, P1 contains the hydrophobic sequence SIEAFCLILLGCITSLI (P1 amino acids 152-168) at its N-terminus which is characteristic for membrane-associated proteins. Thus P1 may associate via this domain with cellular membranes, and in this complex P1-C25 may be produced by proteolytic cleavage and remain associated with this complex by virtue of its nucleic acid-binding capacity (see below).

While monoclonal and/or polyclonal antibodies detected P1 in total protein extracts of PLRV-infected plants (Fig. 2), it was not detectable in subcellular fractions (Fig. 3; data not shown). Since these fractions were prepared under native conditions, it is possible that P1 is rapidly degraded and may, therefore, be only transiently stable as is often observed with autocatalytic polypeptides as in the case of the picornaviruses VPg precursors (2). Also, western analysis of purified PLRV particles with mAbs to the C-terminus of P1 did not detect P1 or P1-C25, indicating that they are not associated with the virion (data not shown).

Characterization of nucleic acid-binding polypeptides derived from the P1 (ORF1) protein

Proteins of PLRV displaying nucleic acid-binding capacity such as P2 (ORF2; 13), P4 (ORF4; 35), and P7 (ORF7; 11)contain a cluster of basic amino acids. The basic motif identified for example in the ORF2 frame of the ORF1/2 transframe protein is responsible for its RNA-binding activity and could possibly represent the RNA template-binding site of the PLRV replicase (13). Computer

analyses of P1 identified a similar stretch of basic amino acid residues (KxKxKKRxRRxxRxK) in the P1 C-terminus (coordinates 1848-1892 in the PLRV-G isolate; D.Prüfer, unpublished). This domain is not part of the VPg coding region or P1/2 (ORF1/2) transframe protein, but nested within the 25 kDa P1-C25 protein.

To test whether the VPg coding region as well as the C-terminal basic domain exhibit the capacity to bind to PLRV RNA, theORF1 deletion derivatives used for the antibody production (Fig. 1B) were subjected to north-western analysis. After separation of bacterial protein extracts by PAGE (Fig. 4A) and electroblotting to nitrocellulose membranes, RNA-binding experiments were performed with radiolabeled single-stranded positive PLRV RNA as described previously (13). Only the fusion protein containing the basic C-terminus (pP1-1; Fig. 4B) exhibited the capacity to bind to PLRV RNA. In a control experiment the ORF0 protein did not display this capacity, as the GST/ORF0 fusion protein (pP0-WT; Fig. 4B) failed to show any binding activity.

This strongly basic region KxKxKKRxRRxxRxK present in P1 and P1-C25 is similar to the cluster of 8-10 amino acids high in lysine and arginine that are present in other nucleic acid-binding proteins (36). Viral proteins with nucleic acid-binding capabilities are often involved in replication and this would suggest a role for P1 and P1-C25 in PLRV multiplication. In fact, for the luteoviruses beet western yellows virus and barley yellow dwarf virus, the luteoviral sequence spanning P1 andP2 has been identified as sufficient for replication in protoplasts (37, 38). Furthermore, earlier studies (13) have shown that the PLRV transframe protein P1/2 as part of the PLRV replicase complex contains both the GDD motif for polymerases as well as a basic nucleic acid-binding domain which resides on the P2 part. Thus P1/2 and P1 (as well as P1-C25) have sequence-unrelated binding domains and probably bind to different PLRV RNA sequences.

By analogy to the sequence of events described for picornaviruses VPg maturation, a model is proposed which involves PLRV P1-C25 and its nucleic acid-binding domain in VPg maturation (Fig. 5). Upon P1 translation from gRNA, the hydrophobic N-proximal P1 domain SIEAFCLILLGCITSLI targets P1 to membrane structures. The basic, hydrophilic C-terminus of P1 binds to PLRV RNA via

its nucleic acid-binding domain such that the VPg sequence and the 5[prime] end of PLRV RNA are juxtaposed. In this membrane-bound complex, proteolytic cleavage of P1 occurs to generate eitherP1-C25* (containing the VPg) or P1-C25 plus proteolytically released VPg with concomitant covalent linkage of VPg to PLRV RNA. In both cases, P1-C25 (or P1-C25*) would remain adjacent to VPg at the 5[prime] end and may play a protective role to prevent VPg proteolysis.

SUMMARY

We have shown by immunological analyses with a set of P1-specific mono- and polyclonal antibodies, the presence of this70 kDa protein in total extracts of PLRV-infected plants. In addition, a 25 kDa protein (P1-C25) that represents the C-terminus of P1 and exhibits nucleic acid-binding capacity, reacts with corresponding antibodies. As deduced from western and computer analyses, the P1-C25 N-terminus either contains the previously identified PLRV VPg or is located in its proximity. A likely role of P1-C25 during VPg maturation is in facilitating replication and represents an excellent point in viral multiplication that can be disrupted and high levels of resistance achieved.

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Figure 1. Schematic diagram of the PLRV genome and of P1 derivatives used in the production of antibodies. (A) Representation of PLRV gRNA. Rectangles represent ORFs. The position of P1 (ORF1) isindicated by shading and occurs in a different reading frame as compared with P0 (ORF0) and P2 (ORF2).(B) Deletion mutants of P1 expressed as pGEX fusion proteins used to prepare monoclonal and polyclonal antibodies. Termini of the fusion proteins are indicated by restriction sites within the coding sequence (A)AluI (coordinate 2160; 8), (P) PstI (coordinate 1950; 8), (S) SwaI (coordinate 1597; 8), (E) EcoRV (coordinate 1165; 8),and (N) NdeI (coordinate 326; 8). Polyclonal antiserum was raised to antigen labelled with a black inverted Y and a grey Y represents mAbs. For pP1-4, monoclonal antibody production was unsuccessful. P1 domains are indicated as follows: hydrophobic domain, hatched rectangle; VPg, grey rectangle; nucleic acid-binding domain, black rectangle (see also Fig. 5).

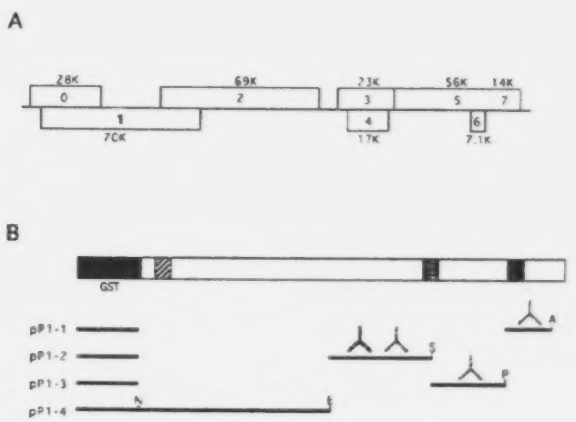


Figure 2. Immunological analyses of P1 and its derivatives in planta. Detection of P1 and P1-C25 in total protein extracts from PLRV-infected *P. floridana* plants using monoclonal (lane b, pP1-1; lane c, pP1-2;and lane d, pP1-3) and polyclonal (pP1-2*) antibodies (lane e). Similar proteins were not detected inextracts from healthy *P. floridana* plants (lanes a and f).

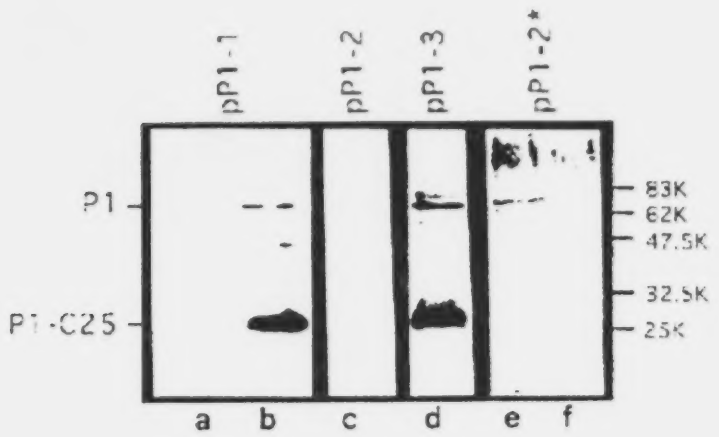


Figure 3. Subcellular localization of P1 and proteolytic P1 products. A western blot was performed with protein extracts from healthy (h) and PLRV-infected *P.floridana* plants (i) and assayed with a mixture of mAbs prepared against pP1-1 and pP1-3. P1-C25 is associated with the membrane fraction (P30), soluble fraction (S30) and cell wall fraction (CW). Immunoreactive proteins of ~18 and 6 kDa, respectively, are indicated by arrows.

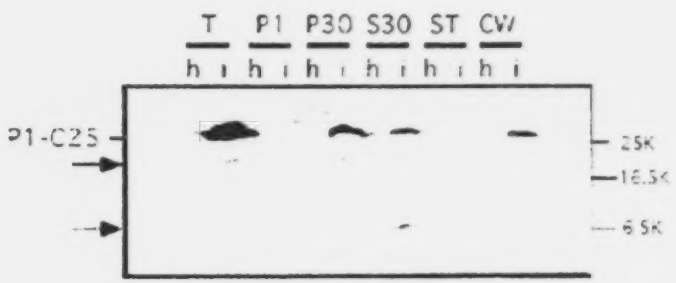


Figure 4. Nucleic acid-binding activity of P1 derivatives. Total protein extracts from bacterial cultures expressing the respective proteins were separated by PAGE on 10% SDS-containing gels and stained with Coomassie Blue (left panel). After electroblotting to nitrocellulose, the membrane was incubated with a32P-labelled PLRV sense RNA. Binding of RNA to pP1-1 protein (indicated by arrows) was visualized by autoradiography.

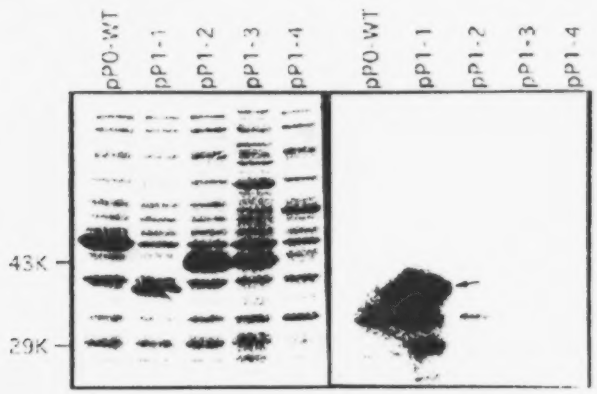
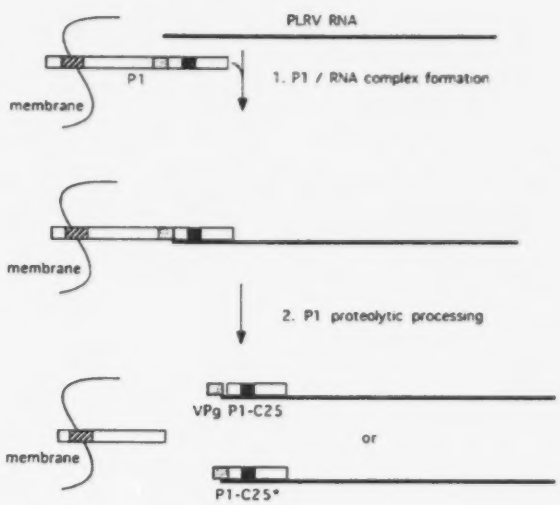


Figure 5. Schematic model of P1 involvement in VPg maturation. A hydrophobic sequence located at the N-terminus of P1 targets the protein to cellular membranes, while a hydrophilic basic nucleic acid-binding domain, located towards the C-terminus, binds to PLRV RNA. This membrane-bound complex serves as a site for VPg maturation with proteolytic processing occurring to release either both P1-C25 and VPg with concomitant covalent VPg linkage to the 5[prime] end of PLRV RNA or VPg as part of P1-C25 (P1-C25*). See legend to Figure 1 for explanation of domains.



Objective B3 - Development of New Crop Protection Strategies

Another strategy being used to develop virus immunity is through reverse genetics. Full-length infectious cDNA clones of German and Canadian potato leafroll luteovirus (PLRV) isolates were developed and examined *in planta* for biological activities. Agroinfection by PLRV cDNA resulted in virus multiplication and systemic movement. Icosohedral PLRV particles were observed with immunosorbent electron microscopy. Differences were observed in infection, movement, and titres of the PLRV produced from the two cDNA clones. This is the first example of PLRV agroinfection and comparison of characteristics between independent infectious cDNA virus clones. Site-directed mutagenesis has already identified unidentified genes absolutely essential to virus replication (D. Prüfer, personal communication). These genes represent alternative strategies to obtaining immunity to PLRV and possibly other viruses.

Potato leafroll virus (PLRV) belongs to the poleroviruseses, one of the most destructive plant viruses. It is transmitted in a circulative manner by aphids and is confined to the phloem tissue of the host plant (Harrison 1984). PLRV particles encapsidate a single-stranded positive-sense RNA of 5.9 kb with a 5' covalently linked VPg (Mayo et al. 1982). Eight major open reading frames (ORFs) are expressed from the genomic RNA (ORF0, ORF1 and ORF2), subgenomic RNA1 (ORF 3, ORF4 and ORF5), or subgenomic RNA2 (ORF6 and ORF7) (Mayo et al. 1989; van der Wilk et al. 1989; Keese et al. 1990; Ashoub et al. 1998). Translation of these various ORFs involves several noncanonical strategies including ribosomal frameshifting and suppression of an amber stop codon (for review see Rohde et al. 1994).

The biological activity of several plant viruses has been examined in protoplasts or transformed plants by introducing full-length viral constructs. Infectious full-length cDNA clones of the luteoviruses beet western yellows (Leiser et al. 1992; Veidt et al. 1992), barley yellow dwarf (Young et al. 1991), cucurbit yellows (Prüfer et al. 1995) and the German (Prüfer et al. 1997) and Canadian (Franco-Lara et al. 1999) isolates of PLRV have also been developed and characterized in protoplasts or transformed plants. In this study, we demonstrate by agroinfection, the biological characteristics

of two independently developed full-length PLRV cDNAs representing isolates from Germany and Canada. Their potential importance in the study of virus infection, multiplication, and movement is described.

The German and Canadian isolates of PLRV selected to prepare cDNA were chosen because they produced high infection levels, severe disease symptoms, and high virus titres. A full-length cDNA clone designated p35SPL-WT ($PLRV_{flg}$) of the German PLRV isolate was constructed from cDNA clones modified by site-directed mutagenesis as described by Prüfer et al. (1997). cDNA clones of a Canadian PLRV isolate (Kawchuk et al. 1989) were modified by *in vitro* mutagenesis and used to prepare the full-length cDNA clone in plasmid p35SUP110 ($PLRV_{flc}$) as described by Franco-Lara et al. (1999). Site-directed mutagenesis was used to add any missing 5' cDNA and remove non-viral sequences. To facilitate transcription, the full-length PLRV constructs are flanked by the CaMV 35S promoter (Guilley et al. 1982) that confers constitutive and high expression of foreign genes in plants (Odell et al. 1985) and the *Agrobacterium tumefaciens nos* terminator. The *E. coli* strain JM109 was used to develop and maintain $PLRV_{fl}$ clones since cell toxicity and cDNA deletions were problematic in other *E.coli* strains (Prüfer, Richards and Kawchuk, unpublished results).

The *A. tumefaciens* strain LB4404 (BRL) was transformed with $PLRV_{flg}$ or $PLRV_{flc}$ DNAs and subsequently introduced in three independent experiments by injection into three leaf midribs (Leiser et al. 1992) of eight, two week old *Nicotiana clevelandii* and *Solanum tuberosum* cv Desiree plants. Plants were maintained in a level two containment facility to exclude aphids and prevent PLRV movement between plants. Uninoculated control plants were included with each experiment to detect external contamination.

Accumulation and movement of PLRV was determined and quantified by indirect ELISA as described by Kawchuk et al. (1990). Samples were taken three and five weeks after inoculation from distal and proximal portions of inoculated and uninoculated leaves. Positive ELISA extracts were examined by immunosorbent electron microscopy with PLRV polyclonal antisera as described by Gill and Chong (1975). Various versions of the BLAST algorithm (Altschul et al. 1997) and

PCGENE software version 6.85 were used to search DNA and protein databases for homology amongst PLRV isolate sequences.

Injection of *A. tumefaciens* LBA4404 carrying full-length cDNA copies indicated that German and Canadian full-length PLRV cDNA were infectious and could be introduced by agroinfection into *N. clevelandii* (Table 1). Injection into the smaller vascular tissues of potato was more difficult and no infection occurred. After 3 and 5 weeks virus was detected by indirect ELISA in both the agroinoculated leaves and in the newly formed upper leaves *of the N. clevelandii plants*. Both $PLRV_{flc}$ and $PLRV_{flg}$ cDNAs behaved in a similar manner in that inoculated primary leaves became infected and PLRV was detected by ELISA three weeks postinoculation. In most inoculated *N. clevelandii* plants, systemic movement was observed and PLRV could be detected by ELISA in newly formed leaves after five weeks. However, in a few cases, the virus was localised within the primarily infected leaf and then was detected in only certain sections of this leaf. Confirmation that the Canadian and German full-length cDNA clones formed virions was obtained by immunosorbent transmission electron microscopy of extracts from agroinfected *N. clevelandii* plants.

Interestingly, strain-specific infectivity was observed with $PLRV_{flc}$ and $PLRV_{flg}$ cDNAs. Infection of the primary agroinoculated and uninoculated leaves occurred at a higher frequency with the $PLRV_{flg}$ cDNA compared to the $PLRV_{flc}$ cDNA (Table 1). Furthermore, viral titres after agroinfection with $PLRV_{flc}$ cDNA were substantially lower in all PLRV ELISA-positive leaves when compared to identical experiments using the $PLRV_{flg}$ cDNA.

Sequence comparisons were performed with GenBank accessions of the Canadian (D13954) and German (submitted) PLRV isolates and defined an overall homology of 97.48% with some unique nucleotide differences among the isolates. Interestingly, the most obvious sequence alterations were found in the overlap region between ORF1 and ORF2 (Fig. 1; nts 1620–2000) (van der Wilk et al. 1989). This region shows a considerable reduction in strain similarity (92.9%) with an exchange of 27 nucleotides leading to an altered amino acid composition of the corresponding proteins.

The molecular analysis of luteovirus infection is difficult because of the relatively low titres of these phloem confined RNA viruses. The most effective strategy to date for characterising the biological function of different luteovirus genes involves "reverse genetics" and full-length cDNA clones into which specific mutations were introduced. For example, the impact of site-directed mutations within luteovirus RNA on replication or disease symptoms can be examined in protoplasts and transgenic plants. However, the strategy of agroinfection facilitates characterisation of these and additional characteristics such as movement in a system that more closely resembles the natural luteovirus infection. Since only a few cells receive T-DNA by agroinoculation, the *in planta* synthesised virus RNA transcripts need to express all proteins necessary for infection, replication, and movement thereby completing the entire virus replication cycle.

Agroinfection was first described as an alternate route of virus infection of plants by Grimsley et al. (1986, 1987) and subsequently applied to the luteoviruses BWYV and CABYV by placing the cDNA under the control of the CaMV 35S promoter (Leiser et al. 1992; Prüfer et al. 1995). Our study describes agroinfection with full-length cDNA clones of PLRV and delineates similarities and differences between infectious cDNA clones for two different PLRV isolates. By analogy to BWYV and CABYV, agroinfection with PLRV clones resulted in replication, movement, and production of virions. However, the efficiency of infection was much lower with $PLRV_{flc}$ and the titres were consistently lower than that observed with $PLRV_{flg}$.

Differences in the severity of disease symptoms produced by various isolates of luteoviruses have been observed (Wright and MacCarthy 1963) and the clones were made to isolates that caused severe disease symptoms. Sequence comparisons of $PLRV_{flc}$ and $PLRV_{flg}$ showed a high level of homology (97.48%) but identified remarkable differences in the ORF1/ORF2 overlap region (Fig. 1) that contains the ribosomal frameshift site for the synthesis of the transframe protein (Prüfer et al. 1992). The proteins encoded by ORF1 and ORF2 are responsible for viral multiplication. Thus one possible explanation for the observed strain differences may be that a more "active" replicase is produced by $PLRV_{flg}$ resulting in a higher virus titer and subsequent level of infection. Indeed, initial experiments replacing this region in the Canadian clone with the corresponding sequence of the German isolate

resulted in a substantial increase in replication efficiency (Jaag et al., in preparation). These results demonstrate that minor sequence differences can influence the infectivity of full-length infectious virus clones and need to be considered when choosing a clone for genetic analysis and biological assays in reverse genetic approaches.

Isolates of PLRV from Australia, Canada, Germany, the Netherlands, Poland and Scotland have been sequenced, but full-length infectious clones were successfully developed only for the two isolates described in this study. Sequence comparisons identified many unique differences in these other PLRV isolates that may affect infectivity. These differences could represent natural virus genomic variations that occur in an infected host or may have resulted from errors in reverse transcription during preparation of cDNA.

In planta infection by full-length PLRV cDNA via agroinfection will overcome several intrinsic difficulties associated with other methods of PLRV expression. Leaf protoplasts, which are used in transient expression experiments, are mainly derived from mesophyll cells, while PLRV is almost entirely confined to cells of the phloem tissue. Similarly, expression of full-length PLRV cDNA in transgenic plants occurs in most cells and does not resemble the conditions of natural infections. Agroinfection represents a strategy that closely resembles natural PLRV infection and is well suited to the *in planta* study of luteovirus proteins and regulatory virus RNA sequences. Agroinfection of infectious full-length PLRV cDNAs as described in this study allows site-directed mutagenesis and the exchange of genes between various luteoviruses to advance our understanding of gene function and host/pathogen interactions.

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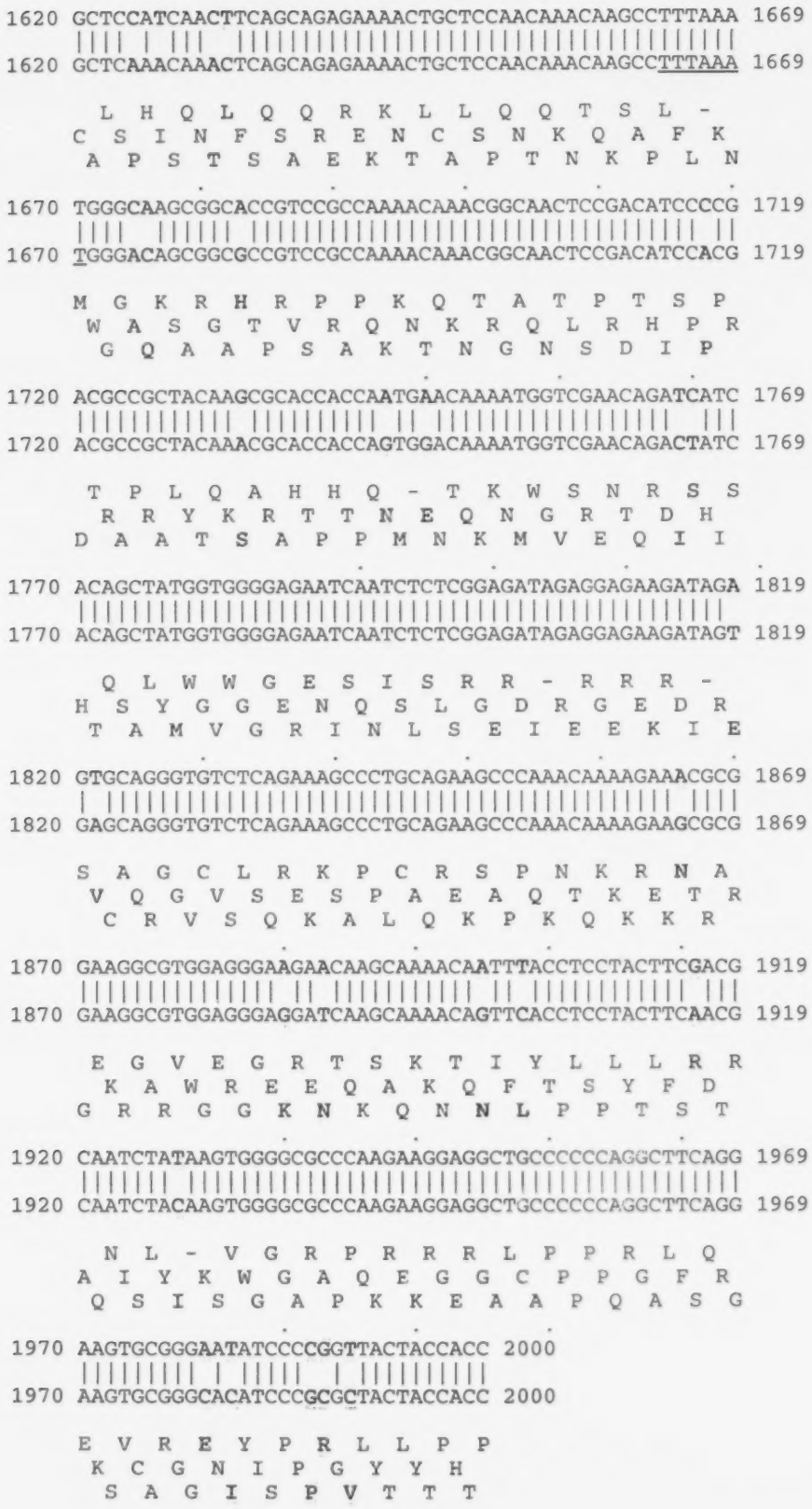
Table 1. Incidence of infection and average PLRV titres and standard error in infected plants. Virus levels were determined in agroinoculated and uninoculated leaves by ELISA (A_{405}).

	Three Weeks		Five Weeks			
	Inoculated (N = 24)		Inoculated (N = 24)		Uninoculated (N = 24)	
	Incidence	Titre	Incidence	Titre	Incidence	Titre
$PLRV_{flg}$	18	0.65 ± 0.32	18	0.54 ± 0.31	18	0.52 ± 0.25
$PLRV_{flc}$	6	0.27 ± 0.07	2	0.29 ± 0.19	2	0.20 ± 0.03

Figure Legend

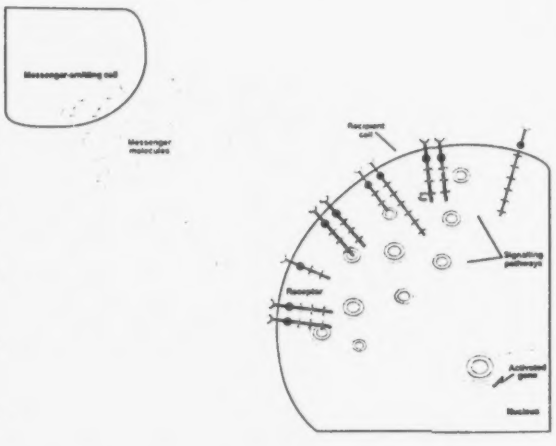
Fig 1. Sequence comparison of the $PLRV_{flc}$ (upper sequence) and $PLRV_{flg}$ (lower sequence) clones in the ORF1/ORF2 overlap region. Location of the ribosomal "slippery sequence" is indicated by underlined nucleotides. Putative amino acid sequences of the $PLRV_{flc}$ are shown below the nucleotide sequences. Sequence differences are shaded.


1620 GCTCCATCAACTTCAGCAGAGAAAACTGCTCCAACAAACAAGCCTTTAAA 1669
1620 GCTCAAACAAACTCAGCAGAGAAAACTGCTCCAACAAACAAGCCTTTAAA 1669
L H Q L Q Q R K L L Q Q T S L -
C S I N F S R E N C S N K Q A F K
A P S T S A E K T A P T N K P L N
1670 TGGGCAAGCGGCACCGTCCGCCAAAACAAACGGCAACTCCGACATCCCCG 1719
1670 TGGGACAGCGGCGCCGTCCGCCAAAACAAACGGCAACTCCGACATCCACG 1719
M G K R H R P P K Q T A T P T S P
W A S G T V R Q N K R Q L R H P R
G Q A A P S A K T N G N S D I P
1720 ACGCCGCTACAAGCGCACCACCAATGAACAAAATGGTCGAACAGATCATC 1769
1720 ACGCCGCTACAAACGCACCACCAGTGGACAAAATGGTCGAACAGACTATC 1769
T P L Q A H H Q - T K W S N R S S
R R Y K R T T N E Q N G R T D H
D A A T S A P P M N K M V E Q I I
1770 ACAGCTATGGTGGGGAGAATCAATCTCTCGGAGATAGAGGAGAAGATAGA 1819
1770 ACAGCTATGGTGGGGAGAATCAATCTCTCGGAGATAGAGGAGAAGATAGT 1819
Q L W W G E S I S R R - R R R -
H S Y G G E N Q S L G D R G E D R
T A M V G R I N L S E I E E K I E
1820 GTGCAGGGTGTCTCAGAAAGCCCTGCAGAAGCCCAAACAAAAGAAACGCG 1869
1820 GAGCAGGGTGTCTCAGAAAGCCCTGCAGAAGCCCAAACAAAAGAAGCGCG 1869
S A G C L R K P C R S P N K R N A
V Q G V S E S P A E A Q T K E T R
C R V S Q K A L Q K P K Q K K R
1870 GAAGGCGTGGAGGGAAGAACAAGCAAAACAATTTACCTCCTACTTCGACG 1919
1870 GAAGGCGTGGAGGGAGGATCAAGCAAAACAGTTCACCTCCTACTTCAACG 1919
E G V E G R T S K T I Y L L L R R
K A W R E E Q A K Q F T S Y F D
G R R G G K N K Q N N L P P T S T
1920 CAATCTATAAGTGGGGCGCCCAAGAAGGAGGCTGCCCCCCAGGCTTCAGG 1969
1920 CAATCTACAAGTGGGGCGCCCAAGAAGGAGGCTGCCCCCCAGGCTTCAGG 1969
N L - V G R P R R R L P P R L Q
A I Y K W G A Q E G G C P P G F R
Q S I S G A P K K E A A P Q A S G
1970 AAGTGCGGGAATATCCCCGGTTACTACCACC 2000
1970 AAGTGCGGGCACATCCCGCGCTACTACCACC 2000
E V R E Y P R L L P P
K C G N I P G Y Y H
S A G I S P V T T T

C. Access to Enabling Technologies

Excellent progress has been made in acquiring enabling and developing technologies that facilitate commercialization of the developed potato varieties. Several promoters have been isolated that provide suitable expression in potato and can be commercialized by AAFC. AAFC has also negotiated a license to a protected transformation vector that may be used in the development of commercial potato varieties. We are using a naked DNA procedure to insert new constructs into potato; this procedure does not require a selection marker. Our objective is to insert a potato, tomato or virus sequence into the potato genome without the need for foreign regulatory sequences - thereby avoiding the impediments to commercialization imposed when utilizing standard marker assisted selection protocols.

The deduced primary structure of *Ve*1 and *Ve*2 included a hydrophobic N-terminal signal peptide, leucine-rich repeats containing 28 or 35 potential glycosylation sites, a hydrophobic membrane-spanning domain, and a cytoplasmic C-terminal domain. These structures suggested that the *Ve* genes encode a novel class of cell surface glycoproteins (see schematic diagram) that could be used as a reporter gene to select transformed protoplasts. Polyclonal antibodies raised against the *Ve* genes have confirmed that the proteins are localised on the cell surface and transformed cells may be selected for plant development. This provides the means of inserting only the desired trait and eliminates the need to use controversial selection markers such as antibiotic resistance.



D. Information Dissemination

Industry Presentations:

The project was outlined to industry stakeholders at the Saskatchewan Seed Potato Growers (SSPGA) Association Annual Field Days held during August of 1998, 1999, 2000 and 2001 in Outlook, SK.

Invited presentations were made to AFIF (Regina, November 2000) and SSPGA (Saskatoon, December, 2000).

Publications and patents:

Kawchuk L.M., Hachey J., Lynch D.R., Kulcsar F., Waterer D.R., Robertson A., Legget, F., Fischer R. and Prufer D. 2002. Mammalian endocytosis signals in plants. Nature (in preparation).

Kawchuk L.M., Hachey J., Lynch D.R., Kulcsar F., van Rooijen G., Waterer D.R., Robertson A., Kokko E., Byers R., Howard R.J., Fischer R. and Prufer D. 2001. Tomato Ve disease resistance genes encode cell surface-like receptors. Proceedings of the National Academy of Sciences U SA 98: 6511-6520.

Kawchuk, L.M. 2001. Potato transformation produces value added traits. *In* "The Handbook of Transgenic Food Plants", G.G. Khachatourians (Ed.), Marcel Dekker Inc. 45: 673-697.

Kawchuk, L.M., Hachey, J., and Lynch, D.R. 1999. Ve protein and nucleic acid sequences, compositions, and methods for plant pathogen resistance. US Patent Application 60/124,129.

Kawchuk, L.M., Hachey, J., and Lynch, D.R. 2000. Ve protein and nucleic acid sequences, compositions, and methods for plant pathogen resistance. PCT Patent Application PCT/CA00/00184.

Kawchuk, L.M., Hachey, J., and Lynch, D.R. 2000. Ve protein and nucleic acid sequences, compositions, and methods for plant pathogen resistance. Colombian Patent Application 00 017.684.

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Kawchuk, L.M., Hachey, J., and Lynch, D.R. 2000. Ve protein and nucleic acid sequences, compositions, and methods for plant pathogen resistance. Philippine Patent Application 1-2000-00530.

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Kawchuk, L.M., Hachey, J., and Lynch, D.R. 2000. Ve protein and nucleic acid sequences, compositions, and methods for plant pathogen resistance. Venezuelan Patent Application 508-00

Personnel on Salary

Dr. L. Hutchison (Research Associate)	Full Time @	$4,000/month
Mr. J. Hachey (Research Technician)	Full Time @	$4,000/month

Budget to Project Completion (March 2002)

Total Funds Allocated to the Project	**$260,275.00**
Total Expenditures	**$260,275.00**
Outstanding Balance	**$ 13,000.00**

Budget Details (attached)

MII Summary by GL Account

FY: 2002
AP: All

A01021 - GEN. ENGINEER POTATO DISEASE

RPT035
Data as of: 2002/04/03

Fund Centre / Fund / Commitment Item / GL Account	Current Year Expended	Prior Year Expended	Current Year Deposits	Prior Year Deposits	Funds Available
A01021 GEN. ENGINEER POTATO DISEASE					
0939 MATCHING INVESTMENT INIT AAFC					
601 SPA DEPOSITS					
20400 SPA/MII Revenue	0.00	0.00	0.00	136,993.00	
610 SPA - SALARY EXPENSE					
20416 SPA/MII SALARY (CSPT)	0.00	58,506.15	0.00	0.00	
20419 SPA/MII SALARY - ALLOWANCES	0.00	756.96	0.00	0.00	
620 SPA - OPERATING EXPENSES					
20428 SPA/MII-POSTAGE,FREIGHT,EXPRESS&CART	69.89	0.00	0.00	0.00	
20436 SPA/MII-PROF&SPEC SERVICES	175.48	0.00	0.00	0.00	
20458 SPA/MII-MAT & SUPPLIES	25,850.06	25,332.00	0.00	0.00	
20482 SPA/MII-MISC EXPENDITURES	4,898.58	0.00	0.00	0.00	
20484 SPA/MII-INT OVER ACCOUNTS	20.13	0.00	0.00	0.00	
630 SPA - MACHINERY AND EQUIPMENT EXPENSES					
20472 SPA/MII-OFFICE EQUIPMENT	15,892.24	0.00	0.00	0.00	
650 DEPOSIT RECEIVED - YEARS TO DATE					
20401 SPA/MII YTD DEPOSITS RECEIVED	0.00	0.00	0.00	123,282.00	
651 SPA -PRIOR YEARS EXPENDITURES					
20414 SPA/MII PRIOR YR EXPENDITURES	0.00	115,588.17	0.00	0.00	
Total MATCHING INVESTMENT INIT AAFC	**46,906.38**	**200,183.28**	**0.00**	**260,275.00**	13,185.34
Total	**46,906.38**	**200,183.28**	**0.00**	**260,275.00**	
TOTAL	**46,906.38**	**200,183.28**	**0.00**	**260,275.00**	

Less Purchase Orders committed and yet to be paid (13,166.88)

18.46 free balance

Certified that this statement is true and correct

Linda Jansen Apr 4/02
Signature Date

~~Incremental and~~ benefits costs ($ 24,525) have been deposited to the Specified Purpose Account reserved for such costs

Linda Jansen Apr 4/02
Signature Date

Rows: 11
Page 1 of 1

Printed 2002/04/0

VE PROTEIN AND NUCLEIC ACID SEQUENCES, COMPOSITIONS, AND METHODS FOR PLANT PATHOGEN RESISTANCE

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of United States Provisional Patent Application Serial No. 60/124,129 filed March 12, 1999 and United States Provisional Patent Application Serial No. 60/130,586 filed April 22, 1999. To the extent that they are consistent herewith, the aforementioned applications are incorporated herein by reference.

FIELD OF THE INVENTION

The invention pertains to the field of molecular biology. In particular, the invention pertains to genes which confer resistance on plants to *Verticillium* species.

BACKGROUND OF THE INVENTION

Verticillium wilt is a common vascular disease that causes severe yield and quality losses in many crops. The disease is caused by fungi of the genus *Verticillium*, most commonly *Verticillium albo-atrum* Reingke & Berthier or *Verticillium dahliae* Kleb. The relationship between *V. albo-atrum* and *V. dahliae* has been the subject of debate (see, generally, Domsch *et al.*, 1980). They have previously been considered representatives of a single variable species, but they have more recently been viewed as distinct species.

V. albo-atrum was first identified as a causal agent of Verticillium wilt in potato, but is now known also to cause wilt in hop, tomato, alfalfa, strawberry, sainfoin, runner bean, broad bean, pea, clover and cucumber. Vascular infection of these hosts by *V. albo-atrum* leads to wilt, with or without obvious flaccidity, and, commonly, browning of the infected xylem stems.

V. dahlia is far more common than *V. albo-atrum*, but causes disease symptoms that are less severe than those caused by *V. albo-atrum* infection. Further, in the above-mentioned hosts, *V. dahlia* is usually less virulent than *V. albo-atrum*. *V. dahlia* is known to infect canola, cotton, dahlia, mint species, vine, tomato, potato, eggplant, olive, pistachio, stone fruits, Brussels sprouts, groundnut, horse radish, tobacco, red pepper, strawberry, and other

plant species. Symptoms of *V. dahlia* infection usually include flaccidity or chlorosis of leaves, followed by permanent wilting.

In a recent survey in North America, Verticillium wilt was ranked as the most important disease of both seed and commercial potato crops, and the second greatest constraint on tuber yield. Pathogen-mediated reductions in net photosynthesis, transpiration, and increased leaf temperature cause premature foliage senescence and yield loss. Disease symptoms in potato include wilting and leaf chlorosis and necrosis, while the tubers of infected plants develop necrosis in the vascular tissue that reduces tuber quality, in particular for the manufacture of french fries and chips. Abiotic factors such as moisture stress and high temperatures accelerate development of visual disease symptoms. Studies have demonstrated a synergistic interaction between root-lesion nematodes (*Pratylenchus penetrans*) and Verticillium wilt, further complicating disease control.

Management strategies for the control of Verticillium wilt include soil fumigation, crop rotation, and development of resistant cultivars. Of these strategies, only disease resistance is effective. While several recent potato cultivar releases have some resistance to the pathogen, the major potato varieties grown in North America are susceptible. The genetic mechanisms of resistance towards fungi of the taxon *Verticillium* spp. vary from polygenic in strawberry and alfalfa, to a dominant single gene in cotton, sunflower, and tomato. Conclusions from studies with tetraploid *Solanum tuberosum* subsp. *tuberosum* L. suggest that inheritance is polygenic and complex. Screening for resistance in the non-cultivated diploid tuber-bearing wild *Solanum* species has identified resistance but not immunity, and a recent study concluded that resistance is polygenic and genetically complex. Thus, incorporation of resistance into new potato cultivars by classical breeding techniques is difficult and inefficient. It would therefore be desirable to develop alternative approaches to obtaining plants that are resistant to Verticillium wilt.

Plants have natural defenses which prevent infection of the plant by viruses, bacteria, fungi, nematodes and insects. As plants do not have a circulatory system, each plant cell must have a preformed or inducible defensive capacity. Recently, disease resistance genes, which confer resistance to specific pathogens, have been identified in various plants. It is believed that the mechanism of resistance may differ depending on the mode of pathogen attack.

Necrotrophy, biotrophy and hemibiotrophy are the three principal modes of pathogen attack on plants (see, generally, Hammond-Kosack *et al.*, 1997). Necrotrophs first kill host cells, then metabolize the cell contents. Necrotrophs often have a broad host range, and cause host cell death with toxins or enzymes targeted to certain substrates. Plant resistance to necrotrophs may therefore be achieved through loss or alteration of the target of the toxin in the plant, or through detoxification of the toxin produced by the plant. An example of a plant disease resistance gene of the latter type *Hm1*, isolated from maize, and which confers resistance to the leaf spot fungus *Cochliobolus carbonum*. *Hm1* encodes a reductase enzyme which is thought to inactivate the *C. carbonum* toxin.

Flor (1949) developed the classic "gene-for-gene" model for plant pathogen resistance. Flor proposed that for incompatibility (i.e. resistance) to occur, complementary pairs of dominant genes are required, one in the host and the other in the pathogen. Loss or alteration of either the plant resistance ("R") gene or the pathogen avirulence ("Avr") gene results in compatibility (i.e. disease). It is thought that R genes encode proteins that can recognize Avr-gene-dependent ligands. The simplest possibility is that the Avr-gene-dependent ligand binds directly to the R gene product. Following binding, the R gene product is believed to activate downstream signaling cascades to induce defense responses, such as the hypersensitive response, which causes localized plant cell death at the point of pathogen attack, thereby depriving the pathogen of living host cells. Downstream signaling components may include kinase and phosphatase cascades, transcription factors, and reactive oxygen species (see, generally, Hammond-Kosack *et al.*, 1997).

Hammond-Kosack *et al.* (1997) summarize the five classes of known R genes, classified according to predicted features of the R gene product.

The first class is composed of R genes that encode detoxifying enzymes. An example is *Hm1* from maize, discussed above, which confers resistance to *Cochliobolus carbonum* (Johal *et al.*, 1992).

R genes of the second class encode intracellular serine/threonine-specific protein kinases. An example is *Pto*, isolated from tomato, and which confers resistance to *Pseudomonas syringe* pv. tomato (Martin *et al.*, 1993).

The third class of R genes is divided into two subclasses. The first encode intracellular proteins with an amino terminal leucine zipper domain, a nucleotide binding site

("NBS") domain, and a leucine rich repeat ("LRR") domain. Examples include *RPS2* of Arabidopsis, which confers resistance to *Pseudomonas syringe* pv. tomato (Bent *et al.*, 1994; Mindrinos *et al.*, 1994), and *I2*, from tomato, which confers resistance to *Fusarium oxysporum* (Ori *et al.*,1997). R genes of the second subclass encode intracellular proteins with an amino terminal domain having homology with *Drosophila* Toll protein, and NBS and LRR domains. Examples include R gene *N* of the tobacco plant, which confers resistance to tobacco mosaic virus (Whitman *et al.*,1994), and *L6* of flax, which confers resistance to *Melampsora lini* (Lawrence *et al.*, 1995).

R genes of the fourth class encode proteins having an extracellular LRR, a single membrane spanning region, and a short cytoplasmic carboxyl terminus. An example is *Cf9* of tomato, which confers resistance to *Cladosporium fulvum* (Jones *et al.*, 1994).

The fifth class encompasses R genes encoding proteins having an extracellular LRR, a single membrane spanning region, and a cytoplasmic kinase domain. An example is R gene *Xa21* of rice, which confers resistance to *Xanthomonas oryzae* pv. *oryzae* (Song *et al.*, 1995).

Certain isolated R genes have been introduced into susceptible plants, resulting in transgenic, disease-resistant plants. For instance, United States Patent No. 5,859,339 teaches transformation of susceptible rice plants with the rice *Xa21* R gene, resulting in transgenic rice plants that are resistant to *Xanthomonas* infection. Unites States Patent No. 5,920,000 teaches transformation of susceptible tomato plants with the tomato *Cf9* R gene, resulting in transgenic tomato plants that are resistant to infection by *Cladosporium fulvum*.

However, the prior art R genes and methods do not confer on plants resistance to *Verticillium* species. Hence, there is a need for isolated Verticillium wilt resistance genes, and for methods for conferring resistance on plant species to infection by *Verticillium* species.

SUMMARY OF THE INVENTION

The invention provides isolated polynucleotides of at least about 50 nucleotides, preferably at least about 100 nucleotides, more preferably at least about 200 nucleotides, more preferably at least about 500 nucleotides, more preferably at least about 1000 nucleotides, and even more preferably at least about 2000 nucleotides, which encode polypeptides comprising amino acid sequences having at least 40% homology to the amino

acid sequence depicted in SEQ ID NO: 2, 4, 6, 8, or 14 and which polynucleotides, when present in a plant, confer on the plant resistance to *Verticillium* species. These polynucleotides are denoted herein as "Ve" polynucleotides. The isolated Ve polynucleotides preferably encode polypeptides comprising amino acid sequences having at least 50% homology, more preferably at least 60% homology, more preferably at least 70% homology, more preferably at least 80% homology, more preferably at least 85% homology, more preferably at least 90% homology, and even more preferably at least 95% homology with the amino acid sequence depicted in SEQ ID NO: 2, 4, 6, 8, or 14. Preferably, the polynucleotides of the invention are isolated from tomato (*Lycopersicon esculentum*), though they may be obtained from other plant species such as potato, hop, alfalfa, strawberry, sainfoin, runner bean, broad bean, pea, clover, cucumber, canola, cotton, dahlia, mint species, vine, eggplant, olive, pistachio, stone fruit, Brussels sprouts, groundnut, horse radish, tobacco, and red pepper.

In an exemplified case, the Ve polynucleotide is isolated from *L. esculentum*, and has the nucleotide sequence depicted in SEQ ID NO: 1 from nucleotide 1 to nucleotide 3417, SEQ ID NO: 3 from nucleotide 57 to nucleotide 3473, SEQ ID NO: 5 from nucleotide 1 to nucleotide 3159, or in SEQ ID NO: 7 from nucleotide 1 to nucleotide 3159. The sequences depicted in SEQ ID NO: 3 from nucleotide 57 to nucleotide 3473, and in SEQ ID NO: 7 from nucleotide 1 to nucleotide 3159 are complementary DNA ("cDNA") sequences, and are denoted herein as, respectively, the *L. esculentum* verticillium wilt resistance genes *Ve1.1* and *Ve1.2*. The sequences depicted in SEQ ID NO: 1 from nucleotide 1 to nucleotide 3417, and SEQ ID NO: 5 from nucleotide 1 to nucleotide 3159 are genomic DNA sequences of *Ve1.1* and *Ve1.2*. *Ve1.1* and *Ve1.2* are two open reading frames ("ORFs") occurring at the same locus, denoted herein as *Ve1*.

In another exemplified case, the Ve polynucleotide is isolated from *Solanum chacoense*, has the partial nucleotide sequence depicted in SEQ ID NO: 13, and is denoted herein as *Vc*.

The invention extends to purified and isolated polynucleotides of at least 50 nucleotides, preferably at least 100 nucleotides, more preferably at least 200 nucleotides, even more preferably at least 500 nucleotides, and most preferably at least 1000 nucleotides, comprising Verticillium wilt resistance gene promoters. An exemplified promoter sequence

is depicted in SEQ ID NO: 10.

The invention also provides nucleic acid constructs, vectors, and transformed cells containing at least one of the aforementioned Ve polynucleotides.

The invention further extends to transgenic plants, cells, seeds and embryos transformed with at least one Ve polynucleotide of the invention, and to methods for conferring on susceptible plants resistance to *Verticillium* species, by transforming plant cells with at least one Ve polynucleotide of the invention and regenerating mature plants. It may be desirable to transform the plant with more than one Ve polynucleotide. For instance, an additive effect may result if the plant is transformed with both *Ve1.1* and *Ve1.2*.

The invention further extends to methods for obtaining a polynucleotide which, when present in a plant, confers on the plant resistance to *Verticillium* species. Broadly stated, such methods comprise the steps of: isolating polynucleotides from a plant having resistance to *Verticillium* species; performing nucleic acid hybridization between said polynucleotides and a probe comprising a nucleotide sequence derived from any of the sequences depicted in SEQ ID NO:1 - 8, 10, 13 or 14; and, testing polynucleotides that hybridize with said probe for ability to confer on a plant resistance to *Verticillium* species.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic genetic and physical representation of the *L. esculentum* linkage group surrounding *Ve1*. Analysis of populations segregating for *Ve1* identified closely linked co-dominant random amplified polymorphic DNAs ("RAPDs") and allele-specific sequence characterized amplified regions ("SCARs") that map to the region of restriction fragment length polymorphism ("RFLP") GP39. Identification of contiguous λ genomic clones facilitated the subcloning of genomic DNA containing the *Ve1* locus. Vertical lines indicate location of the AUG initiation codons. Expressed sequences were cloned into λ and the arrowhead depicts the direction of transcription for the cDNA identified using the genomic clone pG1Ve. Potato plants transformed with the genomic subclones (pG1Ve, pG2Ve, pG3Ve) and cDNA (pC1.1Ve, pC1.2Ve) exhibited *in vivo* complementation and resistance (R) when challenged with *Verticillium albo-atrum.*

Figures 2A and 2B depict the *Ve1.1* genomic DNA sequence (SEQ ID NO: 1, from

nucleotide 1 to nucleotide 3417). The deduced Ve1.1 amino acid sequence encoded by the *Ve1.1* genomic DNA sequence is depicted in SEQ ID NO: 2.

Figures 3A and 3B depict the *Ve1.1* cDNA sequence (SEQ ID NO: 3 from nucleotide 57 to nucleotide 3473).

Figures 4A and 4B depict the primary structure of the encoded Ve1.1 protein (SEQ ID NO: 4) deduced from the *Ve1.1* cDNA sequence (SEQ ID NO: 3 from nucleotide 57 to nucleotide 3473). Domains A through F are identified, as described in Example 1 herein. In the potential membrane associated domains A and C, hydrophobic amino acids are underlined. Within the leucine-rich repeat ("LRR") domain B, conserved amino acids L and G, and potential N-glycosylation sites, are underlined. In domain D, the neutral and basic amino acids are underlined. In domain E, acidic amino acids of the putative PEST sequence are underlined.

Figures 5A and 5B depict the *Ve1.2* genomic DNA sequence (SEQ ID NO: 5 from nucleotide 1 to nucleotide 3159). The deduced Ve1.2 amino acid sequence encoded by the *Ve1.2* genomic DNA sequence is depicted in SEQ ID NO: 6.

Figures 6A and 6B depict the *Ve1.2* cDNA sequence (SEQ ID NO: 7 from nucleotide 1 to nucleotide 3159).

Figure 7 depicts the primary structure of the encoded Ve1.2 protein (SEQ ID NO: 8) deduced from the *Ve1.2* cDNA sequence (SEQ ID NO: 7 from nucleotide 1 to nucleotide 3159). Domains A through E are identified, as described in Example 1 herein. In the potential membrane associated domains A and C, hydrophobic amino acids are underlined. The italicized amino acids in domain A represent the putative leucine zipper region. Within the LRR domain B, conserved amino acids L and G, and potential N-glycosylation sites are underlined. In domain D, the neutral and basic amino acids are underlined. In domain E, acidic amino acids of the putative PEST sequence are underlined.

Figure 8 is a Northern analysis of *Ve1.1* cDNA expression. Hybridization of pC1Ve was determined using 5 µg of poly (A^+) RNA extracted from uninoculated (A) Ailsa Craig and (B) Craigella or (C) Ailsa Craig and (D) Craigella inoculated with race 1 of *V. dahliae* three days prior to RNA isolation. Transcript of *Ve1* is constitutively expressed in susceptible and resistant genotypes.

Figures 9A and 9B are a sequence alignment of the Ve1.1 and Ve1.2 protein

sequences. The sequences have about 84% identity. Aligned identical residues are identified by the "|" character. Aligned similar residues are identified by the "." character. Similar amino acids are deemed to be: A, S, and T; D and E; N and Q; R and K; I, L, M and V; F, Y and W.

Figure 10 depicts a partial genomic DNA sequence of the *Vc* Verticillium wilt resistance gene of *Solanum chacoense* (SEQ ID NO: 13). The deduced partial Vc amino acid sequence encoded by the *Vc* genomic DNA sequence is depicted in SEQ ID NO: 14.

Figures 11A and 11B are a sequence alignment of the Ve1.2 protein sequence and the Vc partial protein sequence. The sequences have about 72% identity. Aligned identical residues are identified by the "|" character. Aligned similar residues are identified by the "." character. Similar amino acids are deemed to be: A, S, and T; D and E; N and Q; R and K; I, L, M and V; F, Y and W.

DETAILED DESCRIPTION OF THE INVENTION

In order to provide a clear and consistent understanding of the specification and claims, including the scope to be given to such terms, the following definitions are provided.

A polynucleotide or polypeptide having the "biological activity" of, respectively, an exemplified Ve polynucleotide or Ve polypeptide of the invention, is a sequence that is functional in a plant to confer resistance to *Verticillium* species, whether to a greater or lesser degree than the subject Ve polynucleotide or Ve polypeptide.

A "coding sequence" is the part of a gene which codes for the amino acid sequence of a protein, or for a functional RNA such as a tRNA or rRNA.

A "complement" or "complementary sequence" is a sequence of nucleotides which forms a hydrogen-bonded duplex with another sequence of nucleotides according to Watson-Crick base-pairing rules. For example, the complementary base sequence for 5'-AAGGCT-3" is 3'-TTCCGA-5'.

A "domain" of a polypeptide is a portion or region of the polypeptide that forms a structural or functional niche within the remainder of the polypeptide. For example, DNA-binding proteins have DNA-binding domains with specific features such as helix-turn-helix configurations or Zn^{2+}-fingers which enable them to recognize and bind to specific structures

or sequences on their target DNA with high specificity and affinity. A "hydrophobic" domain is a domain containing more amino acids having hydrophobic (nonpolar) R groups than amino acids having hydrophilic (polar but uncharged) R groups. A "basic" domain is a domain containing more amino acids having basic R groups than amino acids having acidic R groups. An "acidic" domain is a domain containing more amino acids having acidic R groups than amino acids having basic R groups. The R groups of the 20 amino acids common in proteins are reviewed hereinafter. A domain comprising a plurality of "leucine-rich repeats" is a domain containing more than one repeat of the 24 amino acid consensus sequence depicted in SEQ ID NO: 9.

"Downstream" means on the 3' side of any site in DNA or RNA.

"Expression" refers to the transcription of a gene into structural RNA (rRNA, tRNA) or messenger RNA (mRNA) with subsequent translation into a protein.

Two polynucleotides are "functionally equivalent" if they perform substantially the same biological function. For instance, two polynucleotides are functionally equivalent Verticillium wilt resistance gene promoters if both are functional in a plant to effect the transcription of a Ve polynucleotide operably linked thereto.

Two nucleic acid sequences are "heterologous" to one another if the sequences are derived from separate organisms, whether or not such organisms are of different species, as long as the sequences do not naturally occur together in the same arrangement in the same organism.

Two polynucleotides or polypeptides are "homologous" or "identical" if the sequence of nucleotides or amino acid residues, respectively, in the two sequences is the same when aligned for maximum correspondence as described herein. Sequence comparisons between two or more polynucleotides or polypeptides are generally performed by comparing portions of the two sequences over a comparison window to identify and compare local regions of sequence similarity. The comparison window is generally from about 20 to about 200 contiguous nucleotides or contiguous amino acid residues. The "percentage of sequence identity" or "percentage of sequence homology" for polynucleotides and polypeptides may be determined by comparing two optimally aligned sequences over a comparison window, wherein the portion of the polynucleotide or polypeptide sequence in the comparison window may include additions or deletions (i.e., gaps) as compared to the reference sequence (which

does not comprise additions or deletions) for optimal alignment of the two sequences. The percentage is calculated by: (a) determining the number of positions at which the identical nucleic acid base or amino acid residue occurs in both sequences to yield the number of matched positions; (b) dividing the number of matched positions by the total number of positions in the window of comparison; and, (c) multiplying the result by 100 to yield the percentage of sequence identity.

Optimal alignment of sequences for comparison may be conducted by computerized implementations of known algorithms, or by inspection. A list providing sources of both commercially available and free software is found in Ausubel *et al.* (1999, and in previous editions). Readily available sequence comparison and multiple sequence alignment algorithms are, respectively, the Basic Local Alignment Search Tool (BLAST) (Altschul *et al.*, 1990; Altschul *et al.*, 1997) and ClustalW programs. BLAST is available on the Internet at http://www.ncbi.nlm.nih.gov and a version of ClustalW is available at http://www2.ebi.ac.uk. Other suitable programs include GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package (Genetics Computer Group (GCG), 575 Science Dr., Madison, WI). For greater certainty, as used herein and in the claims, "percentage of sequence identity" or "percentage of sequence homology" of amino acid sequences is determined based on optimal sequence alignments determined in accordance with the default values of the BLASTX program, available as described above.

As discussed in greater detail hereinafter, homology between nucleotide sequences can also be determined by DNA hybridization analysis, wherein the stability of the double-stranded DNA hybrid is dependent on the extent of base pairing that occurs. Conditions of high temperature and/or low salt content reduce the stability of the hybrid, and can be varied to prevent annealing of sequences having less than a selected degree of homology.

"Isolated" means altered "by the hand of man" from the natural state. If an "isolated" composition or substance occurs in nature, it has been changed or removed from its original environment, or both. For example, a polynucleotide or a polypeptide naturally present in a living animal is not "isolated", but the same polynucleotide or polypeptide separated from the coexisting materials of its natural state is "isolated", as the term is employed herein.

Two DNA sequences are "operably linked" if the nature of the linkage does not interfere with the ability of the sequences to effect their normal functions relative to each

other. For instance, a promoter region would be operably linked to a coding sequence if the promoter were capable of effecting transcription of that coding sequence.

A "polynucleotide" is a linear sequence of deoxyribonucleotides (in DNA) or ribonucleotides (in RNA) in which the 3' carbon of the pentose sugar of one nucleotide is linked to the 5' carbon of the pentose sugar of the adjacent nucleotide via a phosphate group.

A "polynucleotide construct" is a nucleic acid molecule which is isolated from a naturally occurring gene or which has been modified to contain segments of nucleic acid which are combined and juxtaposed in a manner which would not otherwise exist in nature.

A "polypeptide" is a linear polymer of amino acids that are linked by peptide bonds.

A "promoter" is a cis-acting DNA sequence, generally 80-120 base pairs long and located upstream of the initiation site of a gene, to which RNA polymerase may bind and initiate correct transcription.

A "recombinant" polynucleotide, for instance a recombinant DNA molecule, is a novel nucleic acid sequence formed in vitro through the ligation of two or more nonhomologous DNA molecules (for example a recombinant plasmid containing one or more inserts of foreign DNA cloned into its cloning site or its polylinker).

A plant that is "resistant" to *Verticillium* species, is a plant that, after inoculation with a strain of *Verticillium*, such as *Verticillium albo-atrum*, exhibits reduced or delayed symptoms of Verticillium wilt, such as wilting, chlorosis, necrosis, and death, relative to a control plant inoculated with the pathogen under the same conditions. A control plant may be, for example, a plant of the same species that has been transformed with a vector that does not have a disease resistance gene inserted therein. Accordingly, a gene that confers "resistance" to *Verticillium* species is a gene that, when present in a plant, makes the plant resistant to *Verticillium* species.

A "Solanaceous plant" is a plant of the botanical family Solanaceae. Also known as the nightshade family, this group includes several widely cultivated plants such as potato, tomato, tobacco, pepper, eggplant, and petunia.

"Transformation" means the directed modification of the genome of a cell by the external application of purified recombinant DNA from another cell of different genotype, leading to its uptake and integration into the subject cell's genome. In bacteria, the recombinant DNA is not integrated into the bacterial chromosome, but instead replicates

autonomously as a plasmid.

A "transgenic" organism, such as a transgenic plant, is an organism into which foreign DNA has been introduced. A "transgenic plant" encompasses all descendants, hybrids, and crosses thereof, whether reproduced sexually or asexually, and which continue to harbour the foreign DNA.

"Upstream" means on the 5' side of any site in DNA or RNA.

A "vector" is a nucleic acid molecule that is able to replicate autonomously in a host cell and can accept foreign DNA. A vector carries its own origin of replication, one or more unique recognition sites for restriction endonucleases which can be used for the insertion of foreign DNA, and usually selectable markers such as genes coding for antibiotic resistance, and often recognition sequences (e.g. promoter) for the expression of the inserted DNA. Common vectors include plasmid vectors and phage vectors.

A "Ve polynucleotide" is a polynucleotide which, when present in a plant, including a transgenic plant, confers on the plant resistance to *Verticillium* species.

A "Ve polypeptide" is a polypeptide encoded by a Ve polynucleotide, and which is active to confer resistance to *Verticillium* species on a plant.

"Verticillium wilt" is a plant disease caused by a fungus of the genus *Verticillium*, and which is characterized by wilting, chlorosis, necrosis, reduced yield, and, possibly, eventual death of the infected plant.

The term "*Verticillium* species" encompasses all members of the genus *Verticillium*, as described by Domsch *et al.* (1980). *Verticillium* species include, without limitation, *V. albo-atrum*, *V. dahlia*, *V. catenulatum*, *V. chlamydosporium*, *V. lecanii*, *V. nigrescens*, *V. nubilum*, *V. psalliotae*, and *V. tricorpus*.

The invention provides Ve polynucleotides which, when present in a plant, confer on the plant resistance to *Verticillium* species. In an exemplified case, the Ve polynucleotide is isolated from *Lycopersicon esculentum*, and has the cDNA sequence depicted in SEQ ID NO: 3 from nucleotide 57 to nucleotide 3473 (*Ve1.1*), or in SEQ ID NO: 7 from nucleotide 1 to nucleotide 3159 (*Ve1.2*) and encodes, respectively, the Ve polypeptide having the amino acid sequence depicted in SEQ ID NOS: 4 or 8. Although it appears that *Ve1.1* and *Ve1.2* do not contain introns, the respective genomic DNA sequences depicted in SEQ ID NO: 1 from nucleotide 1 to nucleotide 3417 and SEQ ID NO: 5 from nucleotide 1 to nucleotide 3159, and

consequently the deduced amino acid sequences depicted in SEQ ID NOS: 2 and 6, differ slightly from the cDNA sequences. Without being limited by the same, it is thought that this difference may be due to natural variation among *L. esculentum* strains. Although both *Ve1.1* and *Ve1.2* are functional to confer on plants resistance to *Verticillium* species, as shown in Figure 9, the *Ve1.1* and *Ve1.2* DNA sequences differ substantially, and have only about 84% sequence homology.

As described in detail in Example 1 herein, *Ve1.1* and *Ve1.2* were isolated from a strain of *L. esculentum* (tomato) that is resistant to *Verticillium dahliae*. Potato plants regenerated from plant cells transformed with a vector containing *Ve1.1* or *Ve1.2*, and then inoculated with *V. albo-atrum*, exhibited reduced and/or delayed symptoms of verticillium wilt (e.g. wilting, chlorosis, necrosis) relative to inoculated control plants transformed only with vector DNA. As shown in Table 1, untransformed potato plants (Désiréee), or potato plants transformed only with vector DNA (pBI121 and pBIN19) exhibited no resistance to *V. albo-atrum*. In contrast, plants transformed with the *Ve1.1* genomic and cDNA sequences (pG3Ve and pC1.1Ve, respectively) and *Ve1.2* genomic and cDNA sequences (pG2Ve and pC1.2Ve, respectively) exhibited substantial resistance to *V. albo-atrum*. Hence, not only do the exemplified Ve polynucleotides confer resistance on various plant species (tomato, potato), they confer resistance to various *Verticillium* species (*V. dahliae* and V. *albo-atrum*).

In another exemplified case, the Ve polynucleotide is isolated from *Solanum chacoense*, a wild potato variety, and has a partial nucleotide sequence as depicted in SEQ ID NO: 13.

It will be appreciated by those of skill in the art that, due to the degeneracy of the genetic code, numerous functionally equivalent nucleotide sequences encode the same amino acid sequence. Therefore, all Ve polynucleotides that encode the Ve polypeptides depicted in SEQ ID NOS: 2, 4, 6, and 8, and the partial Ve polypeptide sequence depicted in SEQ ID NO: 14, are included in the invention.

Further, strains of *L. esculentum* or *S. chacoense* may contain naturally occurring allelic variants of the exemplified Ve polynucleotides. All such allelic variants of the exemplified Ve polynucleotides *Ve1.1* and *Ve1.2*, and the exemplified partial *Vc* sequence, and the encoded Ve polypeptides are included within the scope of the invention.

The invention also extends to truncated Ve polynucleotides and Ve polypeptides that,

despite truncation, retain the ability to confer on plants resistance to *Verticillium* species. For instance, as discussed in Example 1 herein, a fragment of *Ve1.1*, 1332 nucleotides in length (SEQ ID NO: 1 from nucleotide 1 to nucleotide 1332), and a fragment of *Ve1.2*, 1146 nucleotides in length (SEQ ID NO: 5 from nucleotide 1 to nucleotide 1146), were both were functional to confer on a plant resistance to *Verticillium* species.

Using a variety of techniques that are well known in the art (generally as described in Sambrook *et al.*, 1989; Ausubel *et al.*, 1990; Ausubel *et al.*, 1999), the exemplified Ve polynucleotides can be used to isolate additional Ve polynucleotides. Ve polynucleotides of the invention can be isolated from, without limitation, tomato, potato, hop, alfalfa, strawberry, sainfoin, runner bean, broad bean, pea, clover, cucumber, canola, cotton, dahlia, mint species, vine, eggplant, olive, pistachio, stone fruit, Brussels sprouts, groundnut, horse radish, tobacco, and red pepper. In particular, Ve polynucleotides can be isolated from Solanaceous plants. For instance, as discussed in detail in Example 2 herein, the inventors have now mapped a highly active Verticillium wilt resistance gene *Vc* in *Solanum chacoense*, a wild potato variety, to the same position on chromosome IX as *Ve1* occurs in tomato. Further, complete linkage between *Vc* and Verticillium wilt resistance was observed, and *Vc* hybridized with a probe comprising a portion of *Ve1.1*. Moreover, sequence alignment analysis of the Ve1.2 deduced amino acid sequence and a partial deduced amino acid sequence of Vc, having 327 amino acid residues, revealed 72.48% sequence identity. Hence, it appears that *Vc* and *Ve1* may be related Ve polynucleotides.

The Ve polynucleotides depicted in SEQ ID NOS: 1, 3, 5 and 7, and the partial Ve polynucleotide sequence depicted in SEQ ID NO: 13 can be used to construct probes for use in nucleic acid hybridization assays with genomic DNA or complementary DNA ("cDNA") libraries to identify homologous nucleic acid sequences. The principle of hybridization analysis is that a single-stranded DNA or RNA molecule, having a defined sequence, can base-pair with a second DNA or RNA molecule having a complementary sequence to the probe, and which is immobilized. The stability of the probe/target sequence hybrid is dependent on the extent of base pairing that occurs. Techniques for constructing primers and probes, for making recombinant DNA libraries, and for performing nucleic acid hybridization, are well known in the art.

In order to construct a probe, the exemplified Ve polynucleotides can be used first to

design a pair of primers. Alternately, degenerate primers may be designed based on the exemplified Ve polypeptides (SEQ ID NOS: 2, 4, 6 and 8), or the exemplified partial Ve polypeptide (Vc) depicted in SEQ ID NO: 14. The primers are typically obtained by using chemical DNA synthesis to form oligonucleotides of about 30 nucleotides. The primers may be based on any part of the exemplified Ve polynucleotides or Ve polypeptides. Known sequence alignment techniques, as described hereinbefore, can be used to identify conserved regions, which may be preferred sources for primers.

The primers can then used to amplify by polymerase chain reaction ("PCR") a homologous sequence from polynucleotides isolated from a plant that may be either susceptible or resistant to *Verticillium* species. The plant may conveniently be a resistant tomato strain as exemplified herein. The amplified sequence is obtained for subsequent use as a probe in a hybridization assay. Hybridization probes generally have a minimum length of about 200 nucleotides, although smaller probes (e.g. as little as 50 nucleotides in length) can also be used.

To obtain the probe by PCR, double stranded DNA to be amplified is denatured by heating. In the presence of DNA polymerase, excess deoxyribonucleoside triphosphates ("dNTPs"), buffers, salts, and excess single-stranded oligonucleotide primers (based on the exemplified Ve polynucleotides or Ve polypeptides), new DNA synthesis occurs. The primers hybridize to opposite strands of the DNA, and DNA polymerase catalyzes the extension of new strands in the 5' to 3' direction across the DNA segment bounded by the primers. The first cycle of synthesis results in new strands of indeterminate length which, like the parental strands, can hybridize to the primers in the next cycle of denaturation and annealing. These products of indeterminate length build up only arithmetically during subsequent cycles of denaturation, annealing, and DNA synthesis. But discrete products (the target sequence), which are the length of the portion of the parental strands between the 5' ends of the primers, accumulate at an exponential rate, doubling in each cycle of denaturation, annealing and DNA synthesis. Typically, between 20 and 40 cycles are used.

The amplified probe sequence may then be purified by gel electrophoresis. Alternatively, the probe sequence can be cloned into a plasmid and maintained therein, then restricted out of the plasmid and purified by gel electrophoresis. The probe is typically then labeled by, for instance, radio-labeling or biotin-labeling, to permit ready visualization.

The probes based on the exemplified Ve polynucleotide or Ve polypeptide sequences can be used to probe a genetic library of a resistant plant strain. As discussed earlier, a wide variety of plant species may be used as the source of the genetic library. Any resistant strain of a plant species that is known to be affected by Verticillium wilt is a likely candidate for the isolation of Ve polynucleotides. Libraries may be obtained from commercial sources or constructed by known techniques. Genomic libraries are generally constructed by digesting genomic DNA to fragments of manageable size using restriction endonucleases, packaging the genomic DNA fragments into vectors such as bacteriophage λ vectors or cosmid vectors, and introducing the recombinant vectors into suitable host cells (generally *E. coli*). Sufficient numbers of clones are generated to ensure that the particular sequence of interest is represented. The construction of cDNA libraries is similar, but commences with the generation of a double-stranded DNA copy of messenger RNA ("mRNA") from plant tissues of interest through reverse transcription. A primer is annealed to the mRNA, providing a free 3' end that can be used for extension by the enzyme reverse transcriptase. The enzyme engages in the usual 5'-3' elongation, as directed by complementary base pairing with the mRNA template to form a hybrid molecule, consisting of a template RNA strand base-paired with the complementary cDNA strand. After degradation of the original mRNA, a DNA polymerase is used to synthesize the complementary DNA strand to convert the single-stranded cDNA into a duplex DNA.

In a common approach to hybridization analysis, once the appropriate library is constructed, the library is plated out, transferred to a solid support membrane such as a nitrocellulose filter or nylon membrane, and hybridized to the labeled probe.

All hybridization methods (discussed in detail in Sambrook *et al.*, 1989; and in Ausubel *et al.*, 1990, 1999) depend on the ability of denatured DNA to re-anneal when complementary strands are present in an environment near, but below, their melting temperature (T_m), the temperature at which fifty percent of existing DNA duplex molecules are dissociated into single strands. A number of annealing reactions occur during hybridization. These include: annealing of the probe to homologous DNA sequences; mis-matched annealing of the probe to partially homologous sequences; and non-sequence specific interactions, which result in background noise. Mis-matched sequences form less stable hybrids than do completely homologous sequences. As a general rule, the T_m of a

double-stranded DNA molecule decreases by 1 - 1.5°C with every 1% decrease in homology. Increases in temperature, and decreases in salt concentration disfavour annealing, and increase the stringency of the assay. Therefore, hybridization and wash conditions can be adjusted to achieve desired levels of annealing.

Hybridization is typically carried out in solutions of high ionic strength (e.g. 6 × SSC (sodium chloride/sodium citrate buffer) or 6 × SSPE (20 x SSPE = 3.0 M NaCl, 0.2 M NaH2PO4.H2O, 20 mM EDTA, pH 7.4)) at a temperature 20 - 25°C below T_m. For Na^+ concentrations in the range of 0.01 M to 0.4 M, and G + C content from about 30-70%, T_m of hybrids of greater than 100 nucleotides in length can be estimated by the equation Tm = 81.5°C - $16.6(\log_{10}[Na^+])$ + 0.41(%G + C) - 0.63(% formamide) - (600/l), where l = the length of the hybrid in base pairs. This equation applies to the "reversible" T_m defined by measurement of hyperchromicity at OD_{257}. The "irreversible" T_m, which is more important for autoradiographic detection of DNA hybrids is usually 7 - 10°C higher. (Sambrook *et al.*, 1989). A convenient formula for estimating hybridization temperature (T_h) provided in product literature for NYTRAN brand nylon membranes is $T_h = T_m - 5°C = 2°C$ (A-T bp) + 4°C (G-C bp) - 5°C. To avoid background problems, hybridization time and the amount of probe used should be minimized. The probe preferably has a high specific activity and a length of at least about 50 nucleotides.

Washing is performed to remove excess probe, as well as probes that are bound as mis-match hybrids having less than a desired homology level. Washing proceeds in the order from least stringent to most stringent conditions. The stringency of the wash conditions can be varied by adjusting the temperature and salt concentrations of the wash solution. These conditions can be determined empirically by preliminary experiments in which samples of the DNA to be probed are immobilized on filters, hybridized to the probe, and then washed under conditions of different stringencies. By way of illustration, a typical low stringency wash may be conducted at room temperature in a solution of 2 × SSC and 0.1% SDS (sodium dodecyl sulfate). A typical high stringency wash may be conducted at 68°C in a solution of 0.1 × SSC and 0.5% SDS.

Clones that hybridize with the probe at the desired stringency level can then be rescued or isolated, and then sequenced, again using known techniques. As discussed earlier, comparison of the newly isolated sequence with the exemplified Ve polynucleotide sequences

can be performed visually, or by using known algorithms and software packages. Those sequences exhibiting at least 40% homology to the exemplified Ve polynucleotides can then be tested to determine whether they are functional to confer on a susceptible plant resistance to *Verticillium* species. A suitable test is the complementation test described in Example 1 herein. Cells or tissues of a susceptible plant are transformed with the polynucleotide of interest (e.g. as described by De Block, 1988) and transformed plants regenerated. The plants are inoculated with a strain of *Verticillium* which causes Verticillium wilt in the susceptible plant variety. The inoculated plants are examined over an appropriate time course (e.g. at four weeks after inoculation, then at weekly intervals for an additional four weeks) for symptoms of Verticillium wilt. Preferably, control plants such as susceptible non-transformed plants or susceptible plants transformed only with vector DNA are also inoculated with the disease-causing *Verticillium* strain and then monitored for disease symptoms. Delayed or reduced disease symptoms are indicative that the isolated polynucleotide comprises a functional Ve polynucleotide.

The exemplified Ve polynucleotides and Ve polypeptides can be used in conjunction with other known techniques to obtain Ve polynucleotides from genomic DNA, cDNA, RNA, proteins, sequence databases, or plant cells or tissues. For example, using primers derived from the exemplified *Ve1.1* or *Ve1.2* sequences, Ve polynucleotides can be amplified from genomic DNA, cDNA or genomic or cDNA libraries of tomato plants or other plant species (Leister *et al.*, 1996).

In a further alternative method, known immunodetection techniques employing antibodies specific to the Ve1.1 and Ve1.2 polypeptides, or the partial Vc polypeptide, can be used to screen plant cells, tissues, or extracted proteins of interest for the presence of related Ve polypeptides (Sambrook *et al.*, 1989).

Again, such sequences can then be tested by the complementation tests described in Example 1 herein, to determine whether they function to confer on plants resistance to *Verticillium* species.

Additionally, those of skill in the art, through standard mutagenesis techniques, in conjunction with the complementation tests described in Example 1 herein, can obtain altered Ve polynucleotides and test them for the property of conferring on plants resistance to *Verticillium* species. Useful mutagenesis techniques known in the art include, without

limitation, oligonucleotide-directed mutagenesis, region-specific mutagenesis, linker-scanning mutagenesis, and site-directed mutagenesis by PCR (see e.g. Sambrook *et al.*, 1989 and Ausubel *et al.*, 1990, 1999).

In obtaining variant Ve polynucleotides, those of ordinary skill in the art will recognize that proteins may be modified by certain amino acid substitutions, additions, deletions, and post-translational modifications, without loss or reduction of biological activity. In particular, it is well-known that conservative amino acid substitutions, that is, substitution of one amino acid for another amino acid of similar size, charge, polarity and conformation, are unlikely to significantly alter protein function. The 20 standard amino acids that are the constituents of proteins can be broadly categorized into four groups of conservative amino acids on the basis of the polarity of their side chains R- groups) as follows: the nonpolar (hydrophobic) group includes alanine, isoleucine, leucine, methionine, phenylalanine, proline, tryptophan and valine; the polar (uncharged, neutral) group includes asparagine, cysteine, glutamine, glycine, serine, threonine and tyrosine; the positively charged (basic) group contains arginine, histidine and lysine; and the negatively charged (acidic) group contains aspartic acid and glutamic acid. Substitution in a protein of one amino acid for another within the same group is unlikely to have an adverse effect on the biological activity of the protein.

As shown in Figure 9, Ve1.1 and Ve1.2, while both are functional to confer on plants resistance to *Verticillium* species, have sequence identity or homology of only about 84%. Those amino acids that are not identical are likely not essential to protein function. Hence, variation at these amino acids likely will not negatively affect the biological activity of Ve1.2 or Ve1.2. Further, those amino acids that are not identical, but that are similar, likely can be replaced by other similar amino acids, as discussed in the preceding paragraph, without loss of function.

Exemplified Ve polynucleotides isolated from a strain of *Lycopersicon esculentum* which is resistant to *Verticillium dahliae* have been transformed into *Solanum tuberosum* strains that are susceptible to infection by *V. albo-atrum*, resulting in functional complementation. The transformed *Solanum tuberosum* strains displayed reduced disease symptoms following inoculation with *V. albo-atrum* relative to non-transformed plants, illustrating that Ve polynucleotides of the invention are useful for conferring resistance to

various *Verticillium* species on a range of plants. Ve polynucleotides of the invention can also be used to confer resistance to *Verticillium* species in all higher plants which are susceptible to infection by *Verticillium* species, including, without limitation, tomato, potato, hop, alfalfa, strawberry, sainfoin, runner bean, broad bean, pea, clover, cucumber, canola, cotton, dahlia, mint species, vine, eggplant, olive, pistachio, stone fruit, Brussels sprouts, groundnut, horse radish, tobacco, and red pepper.

In preparation for transformation of plant cells with Ve polynucleotides, recombinant vectors are prepared. The desired recombinant vector generally comprises an expression cassette designed for initiating transcription of the Ve polynucleotide in the transformed plant. Additional sequences are included to allow the vector to be cloned in a bacterial or phage host.

The vector will preferably contain a prokaryotic origin of replication having a broad host range. A selectable marker should also be included to allow selection of bacterial cells bearing the desired construct. Suitable prokaryotic selectable markers include resistance to antibiotics such as ampicillin.

Other DNA sequences encoding additional functions may also be present in the vector, as is known in the art. For instance, in the case of *Agrobacterium* mediated transformation, T-DNA sequences will also be included for subsequent transfer to plant chromosomes.

For expression in plants, the recombinant expression cassette will preferably contain, in addition to the desired sequence, a plant promoter region, a transcription initiation site (if the sequence to be transcribed lacks one), and a transcription termination sequence. Unique restriction enzyme sites at the 5' and 3' ends of the cassette are typically included to allow for easy insertion into a pre-existing vector. Sequences controlling eukaryotic gene expression are well known in the art.

The particular promoter used in the expression cassette is not critical to the invention. Any of a number of promoters which direct transcription in plant cells is suitable. The promoter can be either constitutive or inducible. A number of promoters which are active in plant cells have been described in the literature. These include the nopaline synthase (NOS) and octopine synthase (OCS) promoters (which are carried on tumour-inducing plasmids of *Agrobacterium tumefaciens*), the caulimovirus promoters such as the cauliflower mosaic

virus (CaMV) 19S and 35S and the figwort mosaic virus 35S-promoters, the light-inducible promoter from the small subunit of ribulose-1,5-bis-phosphate carboxylase (ssRUBISCO, a very abundant plant polypeptide), and the chlorophyll a/b binding protein gene promoter. All of these promoters have been used to create various types of DNA constructs which have been expressed in plants. A particularly preferred promoter is the endogenous promoter of the Ve polynucleotide. For instance, with respect to *Ve1.1* and *Ve1.2*, the endogenous promoters in SEQ ID NO: 10, are particularly preferred.

The entirety of the endogenous promoter region depicted in SEQ ID NO: 10 need not be used. A relatively short sequence within SEQ ID NO: 10, of as little as 50 nucleotides, may be functional as a promoter, provided that the elements essential to promoter function are included. The promoter region contains sequence of bases that signals RNA polymerase to associate with the DNA, and to initiate the transcription of mRNA using one of the DNA strands as a template to make a corresponding complimentary strand of RNA. Promoter sequence elements include the TATA box consensus sequence (TATAAT), which is usually 20 to 30 base pairs (bp) upstream (by convention -30 to -20 bp relative to the transcription start site) of the transcription start site. In most instances the TATA box is required for accurate transcription initiation. The TATA box is the only upstream promoter element that has a relatively fixed location with respect to the start point. The CAAT box consensus sequence is centered at -75, but can function at distances that vary considerably from the start point and in either orientation. Hence, the TATA box and CAAT box may be within 50 nucleotides of each other. Another common promoter element is the GC box at -90 which contains the consensus sequence GGGCGG. It may occur in multiple copies and in either orientation. Other sequences conferring tissue specificity, response to environmental signals, or maximum efficiency of transcription may also be found in the promoter region. Such sequences are often found within 400 bp of transcription initiation size, but may extend as far as 2000 bp or more. In heterologous promoter/structural gene combinations, the promoter is preferably positioned about the same distance from the heterologous transcription start site as it is from the transcription start site in its natural setting. However, some variation in this distance can be accommodated without loss of promoter function.

Sequences within SEQ ID NO: 10 that provide promoter function can be readily identified. A chimeric construct is created, which includes the fragment of SEQ ID NO: 10 to

be tested for promoter function, operably linked to a reporter gene. Protoplasts are transformed with the chimeric construct, and the expression of the reporter gene is measured. High expression of the reporter gene is indicative of strong promoter function. A suitable reporter gene for the analysis of plant gene expression is the bacterial gene *uidA*, encoding β-glucuronidase ("GUS"). GUS expression can be conveniently quantified through a highly sensitive non-radioactive assay using the fluorogenic substrate 4-MUGluc, as described by Gelvin *et al.* (1994).

In addition to a promoter sequence, the expression cassette preferably also contains a transcription termination region downstream of the structural gene to provide for efficient termination. The termination region may be obtained from the same gene as the promoter sequence, from a different gene, or may be the endogenous termination region of the Ve polynucleotide.

Polyadenylation sequences are also commonly added to the vector construct if the mRNA encoded by the structural gene is to be efficiently translated (Alber *et al.*, 1982). Polyadenylation is believed to have an effect on stabilizing mRNAs. Polyadenylation sequences include, but are not limited to the *Agrobacterium* octopine synthase signal (Gielen *et al.*, 1984) or the nopaline synthase signal (Depicker *et al.*, 1982).

The vector will also typically contain a selectable marker gene by which transformed plant cells can be identified in culture. Typically, the marker gene encodes antibiotic resistance. These markers include resistance to G418, hygromycin, bleomycin, kanamycin, and gentamycin. After transforming the plant cells, those cells containing the vector will be identified by their ability to grow in a medium containing the particular antibiotic.

The recombinant vector is assembled by employing known recombinant DNA techniques (Sambrook *et al.*, 1989; Ausubel *et al.*,1990, 1999). Restriction enzyme digestion and ligation are the basic steps employed to join two fragments of DNA. The ends of the DNA fragment may require modification prior to ligation, and this may be accomplished by filling in overhangs, deleting terminal portions of the fragment(s) with nucleases (e.g., *Exo*III), site directed mutagenesis, or by adding new base pairs by PCR. Polylinkers and adaptors may be employed to facilitate joining of selected fragments. The recombinant vector is typically assembled in stages employing rounds of restriction, ligation, and transformation of *E. coli*.

Recombinant vectors can be introduced into plant cells by a variety of known techniques. Although in the exemplified case potato plant stem and leaf sections were transformed via inoculation with *Agrobacterium tumefaciens* carrying the Ve polynucleotide sequence linked to a binary vector, direct transformation techniques which are known in the art can also be used to transfer the recombinant DNA. For instance, the vector can be microinjected directly into plant cells. Alternatively, nucleic acids may be introduced to the plant cell by high velocity ballistic penetration by small particles having the nucleic acid of interest embedded within the matrix of the particles or on the surface. Fusion of protoplasts with lipid-surfaced bodies such as minicells, cells or lysosomes carrying the DNA of interest can be used. The DNA may also be introduced into plant cells by electroporation, wherein plant protoplasts are electroporated in the presence of plasmids carrying the expression cassette, or by polyethylene glycol ("PEG")-mediated transformation. A review of some of the techniques for incorporating foreign DNA into plant cells is found in Gelvin *et al.* (1994).

In contrast to direct transformation methods, the exemplified case involves vectored transformation using *Agrobacterium tumefaciens*. *Agrobacterium tumefaciens* is a Gram-negative soil bacteria which causes a neoplastic disease known as crown gall in dicotyledonous plants. Induction of tumours is caused by tumour-inducing plasmids known as Ti plasmids. Ti plasmids direct the synthesis of opines in the infected plant. The opines are used as a source of carbon and/or nitrogen by the Agrobacteria.

The bacterium does not enter the plant cell, but transfers only part of the Ti plasmid, a portion called T-DNA, which is stably integrated into the plant genome, where it expresses the functions needed to synthesize opines and to transform the plant cell. *Vir* (virulence) genes on the Ti plasmid, outside of the T-DNA region, are necessary for the transfer of the T-DNA. The *vir* region, however, is not transferred. In fact, the *vir* region, although required for T-DNA transfer, need not be physically linked to the T-DNA and may be provided on a separate plasmid.

The tumour-inducing portions of the T-DNA can be interrupted or deleted without loss of the transfer and integration functions, such that normal and healthy transformed plant cells may be produced which have lost all properties of tumour cells, but still harbour and express certain parts of T-DNA, particularly the T-DNA border regions. Therefore, modified Ti plasmids, in which the disease causing genes have been deleted, may be used as vectors for

the transfer of the Ve polynucleotide constructs of the present invention into plants.

Transformation of plants cells with Agrobacterium and regeneration of whole plants typically involves either co-cultivation of Agrobacterium with cultured isolated protoplasts or transformation of intact cells or tissues with Agrobacterium. In the exemplified case, potato stem and leaf sections were transformed with Agrobacterium.

Alternatively, cauliflower mosaic virus (CaMV) may be used as a vector for introducing DNA into plants of the *Solanaceae* family. For instance, United States Patent No. 4,407,956 to Howell teaches the use of cauliflower mosaic virus DNA as a plant vehicle.

After transformation, transformed plant cells or plants carrying the recombinant DNA are identified. A selectable marker, such as antibiotic resistance, is typically used. In the exemplified case, transformed plant cells were selected by growing the cells on growth medium containing carbenicillin and kanamycin. Other selectable markers will be apparent to those skilled in the art. For instance, the presence of opines can be used to identify transformants if the plants are transformed with Agrobacterium.

Expression of the foreign DNA can be confirmed by detection of RNA encoded by the inserted DNA using well known methods such as Northern blot hybridization. The inserted DNA sequence can itself be identified by Southern blot hybridization or by PCR (see, generally, Sambrook *et al.*, 1989).

Generally, after it is determined that the transformed plant cells carry the recombinant DNA, whole plants are regenerated. Techniques for regenerating differentiated transgenic plants from transformed cells are well known in the art and are described in detail in such references as Gelvin *et al.* (1994). In the exemplified case, potato stem and leaf sections were inoculated with a culture of *Agrobacterium tumefaciens* carrying the desired Ve polynucleotide and carbenicillin and kanamycin marker genes. Transformants were selected on a growth medium containing carbenicillin and kanamycin. After transfer to a suitable medium for shoot induction, shoots were transferred to a medium suitable for rooting. Plants were then transferred to soil and hardened off. The plants regenerated in culture were transplanted and grown to maturity under greenhouse conditions.

The resistance of the regenerated transgenic plants to *Verticillium* species may then be tested. An aggressive isolate of a Verticillium wilt-causing pathogen such as *Verticillium albo-atrum* is isolated from infected plants, and cultured. Transgenic plants containing the

Ve polynucleotide and control plants are then inoculated with a spore suspension of the cultured pathogen. The control plants may be wild type *Verticillium*-sensitive plants, or transgenic plants transformed only with vector DNA which does not contain the Ve polynucleotide insert. Disease reaction ratings can be measured visually, over an appropriate time course. For instance, in potato plants, the first observations may be made four weeks after inoculation, and conducted thereafter at weekly intervals for another four weeks. Plants that show delayed or reduced disease symptoms such as wilting, chlorosis, and necrosis of the leaves, relative to control plants, are rated as being resistant to *Verticillium* species.

Recombinant DNA procedures used for practicing the invention and which are not described in detail herein involve standard laboratory techniques that are well known in the art and are described in standard references such as Sambrook *et al.* (1989) or Ausubel *et al.* (1990, 1999). Generally, enzymatic reactions involving DNA ligase, DNA polymerase, restriction endonucleases and the like are performed according to the manufacturer's specifications. Abbreviations and nomenclature employed herein are standard in the art and are commonly used in scientific publications such as those cited herein.

The invention is further illustrated by the following non-limiting Examples.

EXAMPLE 1

Genetic experiments with near-isogenic lines of *Lycopersicon esculentum* have previously identified a codominant RAPD marker within 3.2 ± 0.3 cM of *Ve1*, a Verticillium wilt resistance gene which confers resistance to race 1 of *Verticillium dahliae* (Kawchuk *et al.*, 1994). Sequences of the RAPD were used to develop SCARs that were determined by high resolution mapping to be within 0.67 ± 0.49 cM or 290 kb of *Ve1* (Kawchuk *et al.*, 1998).

For map-based cloning of *Ve1*, the SCAR sequences were used as hybridization probes to identify λ clones that possess contiguous, overlapping inserts of *Lycopersicon esculentum* VFN8 genomic DNA (Fig. 1). Genomic clones were isolated by screening a λ EMBL3 library (Clonetech Laboratories, Inc., Palo Alto, California, USA) of the *V. dahliae* race 1 resistant *L. esculentum* germplasm VFN8 initially with allele-specific SCARs (GenBank accession Nos. AF029221 and AF029223; Kawchuk *et al.*, 1998). Approximately

three copies of the genome, or 2 x 10^5 recombinant plaques, were transferred to duplicate HYBOND N^+ membranes and probed. Rescued clones were subcloned into pBluescript SK(-) and sequenced.

Identification of *Ve1* involved *in vivo* functional complementation within the *Solanum tuberosum* ssp. *tuberosum* variety Désirée that is highly susceptible to *Verticillium albo-atrum*.

Genomic clones linked to *Ve1* were cloned into the pBIN19 binary vector for *Agrobacterium tumefaciens* mediated transformation. Plant transformation was as described by De Block (1988). Neomycin phosphotransferase II ("NPTII") levels were determined by enzyme-linked immunosorbent assay (Agdia Inc., Elkhart, Indiana, USA) and enzymatic activity measured by radiolabelling of substrate (Staebell, 1990). Plants propagated in the greenhouse were inoculated with *V. albo-atrum* (Kawchuk *et al.*, 1994). Disease reactions were obtained by challenging a minimum of 10 plants from at least three independent lines of transgenic potato plants for each of the pG1Ve, pG2Ve, pG3Ve, and pC1Ve constructs (Fig. 1). *In vivo* complementation was initially observed in potato plants transformed with the 6 kb genomic sequence of λ subclone pG1Ve (Fig. 1).

Referring to Table 1, plants transformed with pG1Ve (comprising, in the 5' to 3' direction, SEQ ID NO: 1 from nucleotide 1 to nucleotide 1332, SEQ ID NO: 10 from nucleotide 1 to nucleotide 3477, and SEQ ID NO: 5 from nucleotide 1 to nucleotide 1146) exhibited a delay and reduced disease symptoms following inoculation with *V. albo-atrum* . Improved complementation was observed in plants transformed with genomic subclone pG2Ve (SEQ ID NO: 5 from nucleotide 1 to nucleotide 3159) or pG3V3 (SEQ ID NO: 1 from nucleotide 1 to nucleotide 3417). In contrast, infection, wilt, chlorosis, necrosis and eventual death of all untransformed plants (Désirée) and plants transformed only with the binary vector DNA (pBI121 or pBIN19) occurred within a few weeks of inoculation.

To identify expressed sequences and the *Ve1* locus, the λ subclones were sequenced, and pG1Ve used to probe a *Lycopersicon esculentum* cDNA library of the Verticillium wilt-resistant variety Craigella. Genomic DNA and cDNA sequences were determined with a BIGDYE Terminator Kit (PE Biosystems, Mississauga, Ontario, Canada) and an ABI 377 automated sequencer (PE Biosystems), using primers derived from the genomic sequences and the polylinker cloning site of the vector. A STRATAGENE (La Jolla, California, USA)

cDNA cloning kit was used to prepare and unidirectionally clone cDNA as described by the manufacturer. Total RNA was isolated from detached leaves of greenhouse propagated *L. esculentum* cultivar Craigella, stressed in 1 mM L-serine for 48 hours. Polyadenylated [poly(A)$^+$] RNA was isolated by oligo(dT) cellulose chromatography. First strand cDNA synthesis was primed with an oligo(dT) linker-primer that contains a *Xho* I site and transcribed using a RNase H$^-$ reverse transcriptase in the presence of 5-methyl dCTP to hemimethylate the cDNA. Second strand cDNA was prepared using RNase H and DNA polymerase I and the double stranded DNA treated with Klenow before ligation to *Eco* RI adapters. The cDNA was ligated to *Eco* RI and *Xho* I restricted arms of the lambda phage vector Uni-ZAP XR vector. Phage were packaged and used to infect the *recA*$^-$ *E. coli* XL1-Blue MRF'. Approximately 3 x 10^5 recombinant plaques were transferred to HYBOND N$^+$ membranes and screened with the genomic subclone pG1Ve. Eight cDNA clones were recovered and the pBluescript SK(-) phagemid with the cloned insert excised and recircularized.

Genomic sequences confirmed that pG1Ve possessed the SCAR sequence linked to the resistant *Ve1* allele and revealed two terminal open reading frames ("ORFs") in pG1Ve homologous to the amino terminal domain of plant and animal receptors that possess leucine-rich repeats. Two cDNA clones, pC1.1Ve (SEQ ID NO: 3 from nucleotide 57 to nucleotide 3473) and pC1.2Ve (SEQ ID NO: 7 from nucleotide 1 to nucleotide 3159), corresponding to the ORFs observed in the genomic subclones were isolated (Fig. 1). Interestingly, the ORF within pG1Ve lacks 751 and 680 amino acids from the C terminus of, respectively, pC1.1Ve (i.e. leaving only amino acids 1 - 388 of SEQ ID NO: 4) and pC1.2Ve (i.e. leaving only amino acids 1 - 382 of SEQ ID NO: 8), demonstrating that *in vivo* complementation can occur without this domain.

To confirm complementation, the cDNA of pC1.1Ve and pC1.2Ve was cloned into the binary vector pBI121 in a sense orientation under transcriptional control of the cauliflower mosaic virus (CaMV) 35S promoter and transformed plants obtained as described for the genomic clones. All plants expressing pC1.1Ve (*Ve1.1*) and pC1.2Ve (*Ve1.2*) exhibited resistance to the pathogen, whereas untransformed germplasm and plants transformed with the vector alone were susceptible and expired within a few weeks of inoculation (Table 1).

Sequence analysis of the cDNA and corresponding genomic clones did not detect any introns within the *Ve1* ORF. As shown in Figures 4A, 4B, and 7, several structural domains were observed within the 1139 and 990 amino acids of Ve1.1 (SEQ ID NO: 4) and Ve1.2 (SEQ ID NO: 8) deduced from the cDNA sequences. Motifs were identified with the PCGENE program (IntelliGenetics Inc.) version 6.85.

Referring to Figures 4A and 4B, six domains, A, B, C, D, E, and F, were identified in Ve1.1 (SEQ ID NO: 4). Domain A is a hydrophobic N terminus of 30 amino acids, indicative of a signal peptide that may target the protein to the cytoplasmic membrane (von Heijne, 1985). Domain B is a LRR with a 24 amino acid consensus XXIXNLXXLXXLXLSXNXLSGXIP (SEQ ID NO: 9) that is often associated with protein-protein interactions and ligand binding. The presence of a glycine within the consensus sequence is consistent with that of extracytoplasmic proteins (Jones, 1994; Song, 1995) and facilitates the recognition of an extracellular pathogen ligand. Thirty five sequences matching the N-glycosylation consensus sequence N(X(S/T) were observed in Ve1.1, within the predicted LRR region. Amino acids in domain C represent a hydrophobic sequence with a predicted α helix secondary structure characteristic of membrane spanning proteins. As frequently observed with type Ia integral membrane proteins, a highly basic region (domain D) follows the hydrophobic domain. Amino acids in domain E include negative residues that define a highly acidic motif similar to PEST sequences observed in cytoplasmic proteins with half-lives of only a few hours (Rogers *et al.*, 1986). The C terminus of Ve1.1 (domain F) concludes with the residues KKF, similar to the KKX motif that functions in animals as a signal for endoplasmic reticulum retention and receptor mediated endocytosis (Jackson *et al.*, 1990).

Referring to Figure 7, domains A, B, E, C and D, corresponding to the similarly identified domains in Ve1.1, were observed in the Ve1.2 amino acid sequence (SEQ ID NO: 8) deduced from the cDNA sequence. Twenty eight sequences matching the N-glycosylation consensus sequence N(X(S/T) were observed in Ve1.2, within the predicted LRR region.

Various versions of the BLAST algorithm (Altschul *et al.*, 1997) were used to search DNA and protein databases for sequences having similarity to Ve1.1 and Ve1.2. Low homology (less than 40% homology, and generally less than 30% homology) to Ve1 was observed in several plant proteins with LRRs such as receptor-like protein kinases

("RLPKs"), antifungal polygalacturonase-inhibiting proteins ("PGIPs"), disease resistance genes that probably produce cytoplasmic proteins, and the genes *Xa* and *Cf* (Jones, 1994; Song, 1995) that appear to produce proteins with an extracytoplasmic domain that interacts with an extracellular ligand. Unlike *Xa*, Ve1 does not include a protein kinase and therefore represents a second member of the *Cf* class of pathogen resistance genes. Although Ve1 structurally resembles the *Cf* resistance genes from tomato, there is little amino acid homology other than the conserved residues of the leucine-rich domain.

Like Ve1, members of the cytokine receptor superfamily posses an extracellular ligand-binding domain, a short single pass transmembrane sequence and a cytoplasmic domain that lacks a kinase motif (Ihle, 1995). Recent crystallographic evidence reveals that hematopoietic and other cytokine receptors are capable of ligand-independent dimerization via ligand binding residues within LRR sequences (Livnah *et al.*, 1999). The unexpected *in vivo* fragment complementation observed with the Ve1 N terminus suggests a similar interaction is occurring with a homologous protein in the susceptible potato plants to produce a heterodimer capable of extracellular ligand recognition and cytoplasmic signaling. This model is supported by the detection of a constitutively expressed Ve1 homolog in susceptible tomato and potato genotypes (Figure 8).

To detect the Ve1 homolog, polyadenylated [poly(A)$^+$] RNA was isolated by oligo(dT) cellulose chromatography from leaves three days post-inoculation, separated on a 1.4% formaldehyde gel, transferred to HYBOND N$^+$ membranes and hybridized with pC1Ve. Heterodimerization between a full-length trans-membrane molecule and a truncated homolog lacking the cytoplasmic domain has been reported to produce a functional bacterial Tar chemoreceptor (Gardena *et al.*, 1996) and monocot *Xa*21 resistance receptor (Wang *et al.*, 1998).

Cytoplasmic signaling by Ve1 may be analogous to that of the erythropoietin cytokine receptor. Preformed dimers on the cell surface facilitate transmission of a ligand-induced conformational change from the extracellular to the cytoplasmic domain and subsequent signal transduction (Remy *et al.*, 1999). The cytoplasmic domain interacts with kinases that link ligand binding to tyrosine phosphoylation of various signaling proteins and transcription activation factors. A similar model has been proposed for the kinase encoded by the *Pto* resistance gene that lacks a receptor domain (Martin, 1993).

Verticillium albo-atrum and *Fusarium oxysporum* are both necrotrophic fungi that invade roots and vascular tissue, and it is somewhat surprising that resistance to these pathogens is conferred by structurally distinct receptors. The *I*2 resistance gene for *F. oxysporum* isolated from tomato (Ori *et al.*, 1997; Simons *et al.*, 1998) resembles the *Arabidopsis* resistance gene *RPS*2 (Bent *et al.*, 1994; Mindrinos *et al.*, 1994) and *RPM*1 (Grant *et al.*, 1995) for *Pseudomonas syringae*. PR proteins of this class are intracellular and possess an N terminal leucine zipper, nucleotide binding site and a leucine rich repeat. Since the *Ve* belongs to the same class of receptors as *Cf*, a gene that confers race-specific resistance to a biotrophic extracellular fungus without haustoria, factors other than infection epidemiology must determine the structure of receptors involved in specific host-pathogen interactions.

Resistance to different pathogen species is contrary to the traditional view of a highly specific interaction with race defining R genes. The results reported herein demonstrate that while the tomato *Ve1* gene has a specificity capable of distinguishing races 1 and 2 of *Verticillium dahliae*, the gene retains the capacity to recognize another *Verticillium* species in a different host. This pleotropic resistance resembles that observed with the *Mi* gene which confers resistance to nematodes and aphids (Milligan *et al.*, 1998; Rossi *et al.*, 1998; Vos *et al.*, 1998) and shares the ability of some R genes to retain biological activity in other plant genera (Rommens *et al.*, 1995; Thilmony *et al.*, 1995; Hammond-Kosack *et al.*, 1998; Whitham, 1996). Several *Verticillium* species infect many agricultural plants, and this pleiotropic host independent complementation by *Ve1* should therefore be of considerable value.

Table 1. Verticillium wilt disease ratings of transformed potato plants at three weeks postinoculation.

Line (N = 30)	Live Plants (%)	Disease Rating*
Desiree	0	5.0 (S)
pBI121	0	5.0 (S)
pBIN19	0	5.0 (S)
pG1Ve	26	4.1 (MS)
pG2Ve	90	0.3 (R)
pG3Ve	53	2.2 (M)
pC1.1Ve	40	2.8 (M)
pC1.2Ve	87	0.4 (R)

* Rating scale is based on percentage of plant exhibiting necrosis and chlorosis: 0 < 20%; 1 = 20 to 40%; 2 = 40 to 60%; 3 = 60 to 80%; 4 = >80%; 5 = 100%. S = susceptible; MS = moderately susceptible; M = moderate; R = resistant.

EXAMPLE 2

A single dominant resistance gene (*Vc*) for verticillium wilt was previously identified in *Solanum chacoense* (Lynch et al. 1997). Herein, we used this segregating population to map *Vc* versus *Ve* in tomato. This was accomplished by restricting extracted progeny and parental DNA with *Taq* I and probing the Southern blots. The Southern blots were probed with *Ve1.1*, and complete linkage was observed with verticillium wilt resistance indicating that not only did *Ve1.1* hybridize to a homologous gene in *S. chacoense* but this gene was also linked to *Vc*.

A potato genomic library (titre 6.8 X 10 PFU/ml) (Clontech, Palo Alto CA, US) of Desiree constructed in EMBL-3 SP6/T7 was probed with *Ve1.1* and three genomic clones isolated. The primary screening was carried out by preparing duplicate plaque lifts of the plated library and probing the supported nitrocellulose membranes with *Ve1.1* labelled with P^{32}. Four washes were performed using 2X SSC/0.1% SDS. Two washes were at 42°C for

15 minutes each and the remaining two washes were at 65°C also for 15 minutes. Plaques yielding positive signals on both membranes were lifted from the plate and eluted into SM buffer. This eluted lambda was used in the secondary screen following the above procedure except that the third and forth washes were performed at 58°C for 15 minutes each. Plaques with a positive signal on duplicate membranes was removed and the lambda eluted. Lambda lysates were prepared from these secondary eluents and DNA was purified from them.

Oligonucleotide primers synthesized to detect *Vel.1* were used to PCR amplify related sequences that may be present in the three rescued genomic clones. Polymerase chain reaction was carried out on the three lambda DNA templates, at various dilutions, to determine if the inserted DNA within these constructs contained the gene of interest. We used primers that were previously generated to construct a *Vel* probe. *Vel* probe primers: 3B2F4 5'- AAT TCA CTC AAC GGG AGC CTT CCT GC - 3' (SEQ ID NO: 11) and 3B2R4-2 5'- TCA AGG CAT TGT TAG AGA AAT CAA G 3' (SEQ ID NO: 12). The reaction mixture contained: 17ul H_2O, 2.5ul 10X PCR buffer, 1.0ul 2.5mM dNTPs, 1.5ul 25mM $MgCl_2$, 0.2ul Amplitaq Gold, 1.0ul primer 3B2F4, 1.0ul primer 3B2R4-2 and 1.0ul template (for a total volume of 25ul). The reaction conditions were 1 cycle at 95°C for 9 min.; 30 cycles at 94°C for 1 min., 60°C for 1 min., and 72°C for 1 min. 30 sec.; and 1 cycle at 72°C for 10 min.. A 15ul aliquot from each reaction tube was loaded onto a 1% agarose gel with EtBr added in and ran at 65 volts for 90 min. A DNA fragment of approximately 850 bp was detected from each genomic clone indicating the presence of a *Vel.1*-related sequence that is most likely *Vc*. Sequencing of this fragment by the methods described in Example 1 herein identified a 982 base pair partial *Vc* genomic sequence having the nucleotide sequence depicted in SEQ ID NO: 13.

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All publications mentioned in this specification are indicative of the level of skill in the art to which this invention pertains. To the extent they are consistent herewith, all publications mentioned in this specification are herein incorporated by reference to the same extent as if each individual publication was specifically and individually indicated to be incorporated by reference.

Although the foregoing invention has been described in some detail by way of illustration and example, for purposes of clarity and understanding it will be understood that certain changes and modifications may be made without departing from the scope or spirit of the invention as defined by the following claims.

WHAT IS CLAIMED IS:

1. A purified and isolated polynucleotide of at least about 50 nucleotides, the presence of which in a plant confers on said plant resistance to *Verticillium* species, said polynucleotide encoding a polypeptide comprising an amino acid sequence having at least 40% homology to the amino acid sequence depicted in SEQ ID NO: 2, SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 8, or to the partial amino acid sequence depicted in SEQ ID NO: 14.

2. The purified and isolated polynucleotide according to claim 1, said polynucleotide encoding a polypeptide comprising an amino acid sequence having at least 50% homology to the amino acid sequence depicted in SEQ ID NO: 2, SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 8, or to the partial amino acid sequence depicted in SEQ ID NO: 14.

3. The purified and isolated polynucleotide according to claim 1, said polynucleotide encoding a polypeptide comprising an amino acid sequence having at least 60% homology to the amino acid sequence depicted in SEQ ID NO: 2, SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 8, or to the partial amino acid sequence depicted in SEQ ID NO: 14.

4. The purified and isolated polynucleotide according to claim 1, said polynucleotide encoding a polypeptide comprising an amino acid sequence having at least 70% homology to the amino acid sequence depicted in SEQ ID NO: 2, SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 8, or to the partial amino acid sequence depicted in SEQ ID NO: 14.

5. The purified and isolated polynucleotide according to claim 1, said polynucleotide encoding a polypeptide comprising an amino acid sequence having at least 80% homology to the amino acid sequence depicted in SEQ ID NO: 2, SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 8, or to the partial amino acid sequence depicted in SEQ

ID NO: 14.

6. The purified and isolated polynucleotide according to claim 1, said polynucleotide encoding a polypeptide comprising an amino acid sequence having at least 90% homology to the amino acid sequence depicted in SEQ ID NO: 2, SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 8, or to the partial amino acid sequence depicted in SEQ ID NO: 14.

7. The purified and isolated polynucleotide according to claim 1, said polynucleotide encoding a polypeptide comprising an amino acid sequence having at least 95% homology to the amino acid sequence depicted in SEQ ID NO: 2, SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 8, or to the partial amino acid sequence depicted in SEQ ID NO: 14.

8. The purified and isolated polynucleotide according to claim 1, said polynucleotide encoding a polypeptide comprising the amino acid sequence depicted in SEQ ID NO: 2, SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 8, or the partial amino acid sequence depicted in SEQ ID NO: 14.

9. The purified and isolated polynucleotide according to claim 1, said polynucleotide comprising the nucleotide sequence depicted in SEQ ID NO: 1 from nucleotide 1 to nucleotide 3417, SEQ ID NO: 3 from nucleotide 57 to nucleotide 3473, SEQ ID NO: 5 from nucleotide 1 to nucleotide 3159, SEQ ID NO: 7 from nucleotide 1 to nucleotide 3159, or the partial nucleotide sequence depicted in SEQ ID NO: 13.

10. The purified and isolated polynucleotide according to claim 1, said polynucleotide being obtained from a tomato, potato, hop, alfalfa, strawberry, sainfoin, runner bean, broad bean, pea, clover, cucumber, canola, cotton, dahlia, mint, vine, eggplant, olive, pistachio, stone fruit, Brussels sprout, groundnut, horse radish, tobacco, or red pepper plant.

11. The purified and isolated polynucleotide according to claim 1, said polynucleotide being obtained from a Solanaceous plant.

12. The purified and isolated polynucleotide according to claim 1, said polynucleotide being obtained from *Lycopersicon* sp.

13. The purified and isolated polynucleotide according to claim 1, said polynucleotide being obtained from *Lycopersicon esculentum*.

14. The purified and isolated polynucleotide according to claim 1, said polynucleotide being obtained from *Solanum* sp.

15. The purified and isolated polynucleotide according to claim 1, said polynucleotide being obtained from *Solanum chacoense*.

16. The purified and isolated polynucleotide according to claim 1, said polynucleotide encoding a polypeptide comprising, in the direction from the amino terminus to the carboxy terminus:

(a) a first hydrophobic domain;

(b) a domain comprising a plurality of leucine-rich repeats;

(c) a second hydrophobic domain;

(d) a basic domain; and,

(e) an acidic domain.

17. The purified and isolated polynucleotide according to claim 1, said polynucleotide encoding a polypeptide comprising, in the direction from the amino terminus to the carboxy terminus:

(a) a first hydrophobic domain;

(b) a domain comprising a plurality of leucine-rich repeats;

(c) an acidic domain;

(d) a second hydrophobic domain; and,

(e) a basic domain.

18. A polynucleotide construct comprising a polynucleotide according to claim 1 operably linked to a plant-expressible promoter.

19. The polynucleotide construct according to claim 18, wherein said promoter is heterologous to said polynucleotide.

20. The polynucleotide construct according to claim 18, wherein said promoter comprises at least 50 nucleotides of the nucleotide sequence depicted in SEQ ID NO: 10.

21. A purified and isolated polynucleotide comprising a Verticillium wilt resistance gene promoter, said promoter comprising at least 50 nucleotides of the nucleotide sequence depicted in SEQ ID NO: 10.

22. The purified and isolated polynucleotide according to claim 21, said polynucleotide comprising the nucleotide sequence depicted in SEQ ID NO: 10.

23. A vector comprising at least one polynucleotide according to claims 1.

24. The vector according to claim 23 wherein said vector is a plant cloning vector.

25. A transgenic plant cell comprising at least one polynucleotide according to claim 1.

26. A transgenic plant, plant seed, or plant embryo, comprising at least one polynucleotide according to any one of claims 1 - 17.

27. A transgenic plant, plant seed, or plant embryo, comprising at least one polynucleotide according to claim 1.

28. The transgenic plant, plant seed, or plant embryo according to claim 27, said plant,

plant seed, or plant embryo being a tomato, potato, hop, alfalfa, strawberry, sainfoin, runner bean, broad bean, pea, clover, cucumber, canola, cotton, dahlia, mint, vine, eggplant, olive, pistachio, stone fruit, Brussels sprout, groundnut, horse radish, tobacco, or red pepper plant, plant seed, or plant embryo.

29. The transgenic plant, plant seed, or plant embryo according to claim 27, said plant, plant seed, or plant embryo being a Solanaceous plant, plant seed, or plant embryo.

30. The transgenic plant, plant seed, or plant embryo according to claim 27, said plant, plant seed, or plant embryo being a potato plant, plant seed, or plant embryo.

31. A method for producing a transgenic plant that is resistant to *Verticillium* species, said method comprising the steps of:

(a) introducing into a plant cell or plant tissue at least one polynucleotide according to any one of claims 1 - 17 to produce a transformed plant cell or plant tissue; and,

(b) regenerating said transformed plant cell or transformed plant tissue to produce said transgenic plant.

32. A method for producing a transgenic plant that is resistant to *Verticillium* species, said method comprising the steps of:

(a) introducing into a plant cell or plant tissue at least one polynucleotide according to claim 1 to produce a transformed plant cell or plant tissue; and,

(b) regenerating said transformed plant cell or transformed plant tissue to produce said transgenic plant.

33. The method according to claim 32, wherein said plant is a tomato, potato, hop, alfalfa, strawberry, sainfoin, runner bean, broad bean, pea, clover, cucumber, canola, cotton, dahlia, mint, vine, eggplant, olive, pistachio, stone fruit, Brussels sprout, groundnut, horse radish, tobacco, or red pepper plant.

34. The method according to claim 32, wherein said plant is a Solanaceous plant.

35. The method according to claim 32, wherein said plant is a potato plant.

36. A method for obtaining a polynucleotide which, when present in a plant, confers on the plant resistance to *Verticillium* species, said method comprising the steps of:

(a) isolating polynucleotides from a plant that is resistant to *Verticillium* species;

(b) performing nucleic acid hybridization between said polynucleotides and a probe comprising a nucleotide sequence derived from any of the sequences depicted in SEQ ID NOS: 1 - 8, 10, 13 or 14; and,

(c) testing the polynucleotides that hybridize with said probe for ability to confer on a plant resistance to *Verticillium* species.

37. The method according to claim 36, wherein said step of performing nucleic acid hybridization is performed under conditions which disfavour annealing of sequences having less than about 40% homology.

38. The method according to claim 36, wherein said testing step comprises the steps of:

(a) transforming plants that are susceptible to *Verticillium* species with polynucleotides that hybridize with said probe;

(b) inoculating the transformed plants with *Verticillium* species, and maintaining the plants under conditions conducive to the establishment of infection by *Verticillium* species; and,

(c) examining the transformed, inoculated plants for symptoms of Verticillium wilt.

39. The method according to claim 36, wherein, in step (a), said plant that is resistant to *Verticillium* species is a tomato, potato, hop, alfalfa, strawberry, sainfoin, runner bean, broad bean, pea, clover, cucumber, canola, cotton, dahlia, mint, vine, eggplant, olive, pistachio, stone fruit, Brussels sprout, groundnut, horse radish, tobacco, or red pepper plant.

40. The method according to claim 36, wherein, in step (a), said plant that is resistant to *Verticillium* species is a Solanaceous plant.

41. The method according to claim 36, wherein, in step (a), said plant that is resistant to *Verticillium* species is a tomato plant.

42. The method according to claim 36, wherein, in step (a), said plant that is resistant to *Verticillium* species is a potato plant.

ABSTRACT

The invention provides polynucleotides which, when present in a plant, confer on the plant resistance to *Verticillium* species. The polynucleotides of the invention are useful for producing transgenic plants that are resistant to *Verticillium* species.

Fig. 1

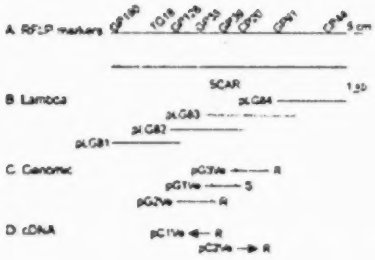
A RFLP markers
CP1/60
TG18
CP1/28
CP23
CP18
CP20
CP21
CP24
5 cm
B Lamboa
SCAR
1.50
pLG84
pLG83
pLG82
pLG81
C Genomic
pG3Ve
R
pG1Ve
S
pG2Ve
R
D cDNA
pC1Ve
R
pC2Ve
R

Fig. 2A

Ve1.1 genomic DNA

ATGAGATTTTTACACTTTCTATGGATCTTCTTCATCATACCCTTTTTGCAAATTTTATTAGG
TAATGAGATTTTATTGGTTTCCTCTCAATGTCTTGATGATCAAAAGTCATTGTTGCTGCAGT
TGAAGGGCAGCTTCCAATATGATTCTACTTTGTCAAATAAATTGGCAAGATGGAACCACAAC
ACAAGTGAATGTTGTAACTGGAATGGGGTTACATGTGACCTCTCTGGTCATGTGATTGCCTT
GGAACTGGATGATGAGAAAATTTCTAGTGGAATTGAGAATGCAAGTGCTCTTTTCAGTCTTC
AGTATCTTGAGAGGCTAAATTTGGCTTACAACAAGTTCAATGTTGGCATACCAGTTGGTATA
GGCAACCTCACCAACTTGACGTACCTGAATTTATCCAATGCCGGTTTTGTTGGCCAAATTCC
TATGATGTTATCAAGGTTAACAAGGCTAGTTACTCTTGATCTCTCAACTCTTTTCCCTGACT
TTGCCCAGCCACTAAAACTAGAGAATCCCAATTTGAGTCATTTCATTGAGAACTCAACAGAG
CTTAGAGAGCTTTACCTTGATGGGGTTGATCTCTCAGCTCAGAGGACTGAGTGGTGTCAATC
TTTATCTTCATATTTGCCTAACTTGACTGTCTTGAGCTTGCGTACTTGTCGAATTTCAGGCC
CTATTGATGAATCACTTTCTAAGCTTCACTTTCTCTCTTTCATCCGTCTTGACCAGAACAAT
CTCTCTACCACAGTTCCTGAATACTTTGCCAATTTCTCAAACTTGACTACCTTGACCCTCTC
CTCTTGTAATCTGCAAGGAACATTTCCTAAAAGAATCTTTCAGGTACCAGTCTTAGAGTTTT
TGGACTTGTCAACTAACAAATTGCTTAGTGGTAGTATTCCGATTTTTCCTCAAATTGGATCA
TTGAGGACGATATCACTAAGCTACACCAAGTTTTCTGGTTCATTACCAGACACCATTTCGAA
CCTTCAAAACCTATCCAGGTTAGAACTCTCCAACTGCAATTTCAGTGAACCAATACCTTCCA
CAATGGCGAACCTTACCAATCTTGTTTATTTAGATTTCTCCTTCAACAATTTCACTGGTTCC
CTCCCATATTTCCAAGGGGCCAAGAAACTCATCTACTTGGACCTTTCACGTAATGGTCTAAC
TGGTCTCTTGTCTAGAGCTCATTTTGAAGGACTCTCAGAACTTGTCTACATTAATTTAGGGA
ACAATTCACTCAACGGGAGCCTTCCTGCATATATATTTGAGCTCCCCTCGTTGAAGCAGCTT
TTTCTTTACAGCAATCAATTTGTTGGCCAAGTCGACGAATTTCGCAATGCATCCTCCTCTCC
GTTGGATACAGTTGACTTGAGAAACAACCACCTGAATGGATCGATTCCCAAGTCCATGTTTG
AAGTTGGGAGGCTTAAGGTCCTCTCACTTTCTTCCAACTTCTTTAGAGGGACAGTTCCCCTT
GACCTCATTGGGAGGCTGAGCAACCTTTCAAGACTGGAGCTTTCTTACAATAACTTGACTGT
TGATGCAAGTAGCAGCAATTCAACCTCTTTCACATTTCCCCAGTTGAACATATTGAAATTAG
CGTCTTGTCGGCTGCAAAAGTTCCCCGATCTCAAGAATCAGTCAAGGATGATGCACTTAGAC
CTTTCAGACAACCAAATATTGGGGGCAATACCAAATTGGATCTGGGGAATTGGTGGTGGAGG
TCTCGCCCACCTGAATCTTTCATTCAATCAGCTGGAGTACGTGGAACAGCCTTACACTGTTT
CCAGCAATCTTGCAGTCCTTGATTTGCATTCCAACCGTTTAAAAGGTGACTTACTAATACCA
CCTTCCACTGCCATCTATGTGGACTACTCGAGCAATAATTTAAACAATTCCATCCCAACAGA
TATTGGAAGATCTCTTGGTTTTGCCTCCTTTTTCTCGGTAGCAAACAATAGCATCACTGGAA
TAATTCCTGAATCCATATGCAACGTCAGCTACCTTCAAGTTCTTGATTTCTCTAACAATGCC
TTGAGTGGAACAATACCACCATGTCTACTGGAATATAGTCCAAAACTTGGAGTGCTGAATCT
AGGGAACAATAGACTCCATGGTGTTATACCAGATTCATTTCCAATTGGTTGTGCTCTAATAA
CTTTAGACCTCAGCAGGAATATCTTTGAAGGGAAGCTACCAAAATCGCTTGTCAACTGCACG
TTGTTGGAGGTCCTGAATGTTGGAAATAACAGTCTTGTTGATCGTTTCCCATGCATGTTGAG
GAACTCAACCAGCCTGAAGGTCCTAGTCTTGCGCTCCAATAAATTCAATGGAAATCTTAC

Fig. 2B

GTGTAATATAACCAAACATAGCTGGAAGAATCTCCAGATCATAGATATAGCTTCCAACAATT
TTACTGGTATGTTGAATGCAGAATGCTTTACAAATTGGAGAGGAATGATGGTTGCAAAAGAT
TACGTGGAGACAGGACGCAATCATATCCAGTATGAGTTCTTACAACTAAGTAACTTGTACTA
TCAGGATACAGTGACATTAATCATCAAAGGCATGGAGCTGGAGCTTGTGAAGATTCTTAGGG
TCTTCACATCTATTGATTTCTCTTCCAATAGATTTCAAGGAAAGATACCAGATACTGTTGGG
GATCTTAGCTCACTTTATGTTTTGAACCTGTCACACAATGCCCTCGAGGGACCAATTCCAAA
ATCAATTGGGAAGCTACAAATGCTTGAATCACTAGACCTGTCAACAAACCACCTGTCCGGGG
AGATCCCCTCAGAGCTTTCAAGTCTCACATTCTTAGCAGTTTTGAACTTATCGTTCAACAAT
TTGTTTGGAAAAATCCCGCAAAGTAATCAATTTGAAACATTCCCAGCAGAATCCTTTGAAGG
AAACAGAGGCCTATGCGGGCTTCCTCTTAACGTCATTTGCAAAAGCGATACTTCAGAGTTGA
AACCAGCACCAAGTTCTCAAGATGACTCTTATGATTGGCAGTTCATATTTACGGGTGTGGGA
TATGGAGTAGGGGCAGCAATCTCCATTGCACCTCTGTTGTTTTACAAGCAAGGAAACAAATA
CTTTGACAAACATTTGGAGAGAATGCTTAAACTGATGTTTCCTAGATACTGGTTCAGTTACA
CCAGATTTGACCCTGGGAAGGTTGTGGCTGTGGAACACTATGAAGATGAGACCCCAGATGAC
ACCGAAGATGACGATGAGGGGGGAAAAGAAGCATCTCTTGGGCGTTATTGTGTCTTCTGTAG
TAAACTTGATTTTCAGAAAAATGAAGCAATGCATGATCCAAAATGCACTTGTCATATGTCAT
CATCCCCCAATTCTTTTCCTCCTACGCCGTCCTCTTCTTCACCTTTATTAGTCATATATCAC
AAAAAGTTTTGA

Fig. 3A

Ve1.1 cDNA

GCACGAGAGAAAAAACAACAAGTTTGATGGATTATAATTCCTCCAAGACTTAAGCAATGAGA
TTTTTACACTTTCTATGGATCTTCTTCATCATACCCTTTTTGCAAATTTTATTAGGTAATGA
GATTTTATTGGTTTCCTCTCAATGTCTTGATGATCAAAAGTCATTGTTGCTGCAGTTGAAGG
GCAGCTTCCAATATGATTCTACTTTGTCAAATAAATTGGCAAGATGGAACCACAACACAAGT
GAATGTTGTAACTGGAATGGGGTTACATGTGACCTCTCTGGTCATGTGATTGCCTTGGAACT
GGATGATGAGAAAATTTCTAGTGGAATTGAGAATGCAAGTGCTCTTTTCAGTCTTCAGTATC
TTGAGAGGCTAAATTTGGCTTACAACAAGTTCAATGTTGGCATACCAGTTGGTATAGGCAAC
CTCACCAACTTGACGTACCTGAATTTATCCAATGCCGGTTTTGTTGGCCAAATTCCTATGAT
GTTATCAAGGTTAACAAGGCTAGTTACTCTTGATCTCTCAACTCTTTTCCCTGACTTTGCCC
AGCCACTAAAACTAGAGAATCCCAATTTGAGTCATTTCATTGAGAACTCAACAGAGCTTAGA
GAGCTTTACCTTGATGGGGTTGATCTCTCAGCTCAGAGGACTGAGTGGTGTCAATCTTTATC
TTCATATTTGCCTAACTTGACTGTCTTGAGCTTGCGTACTTGTCGAATTTCAGGCCCTATTG
ATGAATCACTTTCTAAGCTTCACTTTCTCTCTTTCATCCGTCTTGACCAGAACAATCTCTCT
ACCACAGTTCCTGAATACTTTGCCAATTTCTCAAACTTGACTACCTTGACCCTCTCCTCTTG
TAATCTGCAAGGAACATTTCCTAAAAGAATCTTTCAGGTACCAGTCTTAGAGTTTTTGGACT
TGTCAACTAACAAATTGCTTAGTGGTAGTATTCCGATTTTTCCTCAAATTGGATCATTGAGG
ACGATATCACTAAGCTACACCAAGTTTTCTGGTTCATTACCAGACACCATTTCGAACCTTCA
AAACCTATCCAGGTTAGAACTCTCCAACTGCAATTTCAGTGAACCAATACCTTCCACAATGG
CGAACCTTACCAATCTTGTTTATTTAGATTTCTCCTTCAACAATTTCACTGGTTCCCTCCCA
TATTTCCAAGGGGCCAAGAAACTCATCTACTTGGACCTTTCACGTAATGGTCTAACTGGTCT
CTTGTCTAGAGCTCATTTTGAAGGACTCTCAGAACTTGTCTACATTAATTTAGGGAACAATT
CACTCAACGGGAGCCTTCCTGCATATATATTTGAGCTCCCCTCGTTGAAGCAGCTTTTTCTT
TACAGCAATCAATTTGTTGGCCAAGTCGACGAATTTCGCAATGCATCCTCCTCTCCGTTGGA
TACAGTTGACTTGAGAAACAACCACCTGAATGGATCGATTCCCAAGTCCATGTTTGAAGTTG
GGAGGCTTAAGGTCCTCTCACTTTCTTCCAACTTCTTTAGAGGGACAGTTCCCCTTGACCTC
ATTGGGAGGCTGAGCAACCTTTCAAGACTGGAGCTTTCTTACAATAACTTGACTGTTGATGC
AAGTAGCAGCAATTCAACCTCTTTCACATTTCCCCAGTTGAACATATTGAAATTAGCGTCTT
GTCGGCTGCAAAAGTTCCCCGATCTCAAGAATCAGTCAAGGATGATGCACTTAGACCTTTCA
GACAACCAAATATTGGGGGCAATACCAAATTGGATCTGGGGAATTGGTGGTGGAGGTCTCGC
CCACCTGAATCTTTCATTCAATCAGCTGGAGTACGTGGAACAGCCTTACACTGTTTCCAGCA
ATCTTGTAGTCCTTGATTTGCATTCCAACCGTTTAAAAGGTGACTTACTAATACCACCTTCC
ACTGCCATCTATGTGGACTACTCGAGCAATAATTTAAACAATTCCATCCCAACAGATATTGG
AAGATCTCTTGGTTTTGCCTCCTTTTTCTCGGTAGCAAACAATAGCATCACTGGAATAATTC
CTGA

Fig. 3B

ATCCATATGCAACGTCAGCTACCTTCAAGTTCTTGATTTCTCTAACAATGCCTTGAGTGGAA
CAATACCACCATGTCTACTGGAATATAGTCCAAAACTTGGAGTGCTGAATCTAGGGAACAAT
AGACTCCATGGTGTTATACCAGATTCATTTCCAATTGGTTGTGCTCTAATAACTTTAGACCT
CAGCAGGAATATCTTTGAAGGGAAGCTACCAAAATCGCTTGTCAACTGCACGTTGTTGGAGG
TCCTGAATGTTGGAAATAACAGTCTTGTTGATCGTTTCCCATGCATGTTGAGGAACTCAACC
AGCCTGAAGGTCCTAGTCTTGCGCTCCAATAAATTCAATGGAAATCTTACGTGTAATATAAC
CAAACATAGCTGGAAGAATCTCCAGATCATAGATATAGCTTCCAACAATTTTACTGGTATGT
TGAATGCAGAATGCTTTACAAATTGGAGAGGAATGATGGTTGCAAAAGATTACGTGGAGACA
GGACGCAATCATATCCAGTATGAGTTCTTACAACTAAGTAACTTGTACTATCAGGATACAGT
GACATTAATCATCAAAGGCATCGAGCTGGAGCTTGTGAAGATTCTTAGGGTCTTCACATCTA
TTGATTTCTCTTCCAATAGATTTCAAGGAAAGATACCAGATACTGTTGGGGATCTTAGCTCA
CTTTATGTTTTGAACCTGTCACACAATGCCCTCGAGGGACCAATTCCAAAATCAATTGGGAA
GCTACAAATGCTTGAATCACTAGACCTGTCAAGAAACCACCTGTCCGGGGAGATCCCCTCAG
AGCTTTCAAGTCTCACATTCTTAGCAGTTTTGAACTTATCGTTCAACAATTTGTTTGGAAAA
ATCCCGCAAAGTAATCAATTTGAAACATTCTCAGCAGAATCCTTTGAAGGAAACAGAGGCCT
ATGCGGGCTCCCTCTTAACGTCATTTGCAAAAGCGATACTTCAGAGTTGAAACCAGCACCAA
GTTCTCAAGATGACTCTTATGATTGGCAGTTCATATTTACGGGTGTGGGATATGGAGTAGGG
GCAGCAATCTCCATTGCACCTCTGTTGTTTTACAAGCAAGGAAACAAATACTTTGACAAACA
TTTGGAGAGAATGCTTAAACTGATGTTTCCTAGATACTGGTTCAGTTACACCAGATTTGACC
CTGGGAAGGTTGTGGCTGTGGAACACTATGAAGATGAGACCCCAGATGACACCGAAGATGAC
GATGAGGGTGGAAAAGAAGCATCTCTTGGGCGTTATTGTGTCTTCTGTAGTAAACTTGATTT
TCAGAAAAATGAAGCAATGCATGATCCAAAATGCACTTGTCATATGTCATCATCCCCCAATT
CTTTTCCTCCTACGCCGTCCTTTTTTTCACCTTTATTAGTCATATATCACAAAAAGTTTTGA
TT

Fig. 4A

Ve1.1 AA from cDNA

```
A                MRFLHFLWIFFIIPFLQILLGNEILLVSSQ                30

B                    CLDDQKSLLLQLKGSFQYDSTLSNKLA                60
         RWNHNTSECCNWNGVTCDLSGHVIALELDDEKISSGIE                 95
                     NASALFSLQYLERLNLAYNKFNVGIP                121
                       VGIGNLTNLTYLNLSNAGFVGQIP                145
                       MMLSRLTRLVTLDLSTLFPDFAQP                169
                LKLENPNLSHFIENSTELRELYLDGVDLSAQRT              202
                     EWCQSLSSYLPNLTVLSLRTCRISGPID              230
                        ESLSKLHFLSFIRLDQNNLSTTVP               254
                        EYFANFSNLTTLTLSSCNLQGTFP               278
                        KRIFQVPVLEFLDLSTNKLLSGSIP              303
                          IFPQIGSLRTISLSYTKFSGSLP               326
                        DTISNLQNLSRLELSNCNFSEPIP               350
                        STMANLTNLVYLDFSFNNFTGSLP               374
                         YFQGAKKLIYLDLSRNGLTGLLS               397
                       RAHFEGLSELVYINLGNNSLNGSLP               422
                        AYIFELPSLKQLFLYSNQFVGQVD               446
                       EFRNASSSPLDTVDLRNNHLNGSIP               471
                        KSMFEVGRLKVLSLSSNFFRGTVP               495
                       LDLIGRLSNLSRLELSYNNLTVDAS               520
                     SSNSTSFTFPQLNILKLASCRLQKFPD               547
                          LKNQSRMMHLDLSDNQILGAIP               569
                      NWIWGIGGGGLAHLNLSFNQLEYVEQ               595
                         PYTVSSNLVVLDLHSNRLKGDLLIP             620
                              PSTAIYVDYSSNNLNNSIP               639
                       TDIGRSLGFASFFSVANNSITGIIP               664
                        ESICNVSYLQVLDFSNNALSGTIP               688
                       PCLLEYSPKLGVLNLGNNRLHGVIP               713
                        DSFPIGCALITLDLSRNIFEGKLP               737
                        KSLVNCTLLEVLNVGNNSLVDRFP               761
                        CMLRNSTSLKVLVLRSNKFNGNLT               785
                      CNITKHSWKNLQIIDIASNNFTGMLN               811
                       AECFTNWRGMMVAKDYVETGRNHIQ               836
                           YEFLQLSNLYYQDTVTLIIKG               857
                     MELELVKILRVFTSIDFSSNRFQGKIP               884
                        DTVGDLSSLYVLNLSHNALEGPIP               908
                        KSIGKLQMLESLDLSRNHLSGEIP               932
                        SELSSLTFLAVLNLSFNNLFGKIP               959
                QSNQFETFSAESFEGNRGLCGLPLNVICKSDTSE             990

C             LKPAPSSQDDSYDWQFIFTGVGYGVGAAISIA                1022
```

Fig. 4B

D	PLLFYKQGNKYFDKHLERMLKLMFPRYWFSYTRFDPGKVVAV	1064
E	EHYEDETPDDTEDDDEGG	1082
F	KEASLGRYCVFCSKLDFQKNEAMHDPKCTC	1112
	HMSSSPNSFPPTPSFFSPLLVIYHKKF	1139

Fig. 5A

Ve1.2 genomic DNA

ATGAAAATGATGGCAACTCTGTACTTCCCTATGGTTCTCTTGATTCCCTCGTTTCAAATCTT
ATCAGGATACCACATTTTCTTGGTTTCCTCTCAATGCCTTGACGATCAAAAGTCATTGTTGC
TGCAGTTTAAGGGAAGCCTCCAATATGATTCTACTTTGTCAAAGAAATTGGCAAAATGGAAC
GACATGACAAGTGAATGTTGCAATTGGAATGGGGTTACATGCAATCTCTTTGGTCATGTGAT
CGCTTTGGAACTGGATGATGAGACTATTTCTAGTGGAATTGAGAATTCTAGTGCACTTTTCA
GTCTTCAATATCTTGAGAGCCTAAATTTGGCTGACAACATGTTCAATGTTGGCATACCAGTT
GGTATAGACAACCTCACAAACTTGAAGTACCTGAATTTATCCAATGCTGGTTTTGTCGGGCA
AATTCCTATAACATTATCAAGATTAACAAGGCTAGTTACTCTTGATCTCTCAACTATTCTCC
CTTTTTTTGATCAGCCACTTAAACTTGAGAATCCCAATTTGAGTCATTTCATTGAGAACTCA
ACAGAGCTTAGAGAGCTTTACCTTGATGGGGTTGATCTTTCGTCTCAGAGGACTGAGTGGTG
TCAATCTTTATCTTTACATTTGCCTAACTTGACCGTTTTGAGCTTGCGTGATTGTCAAATTT
CAGGCCCTTTGGATGAATCACTTTCTAAGCTTCACTTTCTCTCTTTTGTCCAACTTGACCAG
AACAATCTCTCTAGCACAGTTCCTGAATATTTTGCCAATTTCTCGAACTTGACTACATTGAC
CCTGGGCTCTTGTAATCTACAGGGAACATTTCCTGAAAGAATCTTTCAGGTATCAGTTTTAG
AGAGTTTGGACTTGTCAATTAACAAGTTGCTTCGTGGTAGTATTCCAATTTTTTTCCGAAAT
GGATCTCTGAGGAGGATATCACTAAGCTACACCAACTTTTCCGGTTCATTACCAGAGTCCAT
TTCGAACCATCAAAATCTATCCAGGTTAGAGCTTTCTAATTGCAATTTCTATGGATCAATAC
CTTCCACAATGGCAAACCTTAGAAATCTTGGTTATTTGGATTTCTCCTTCAACAATTTCACT
GGTTCTATCCCATATTTTCGACTGTCCAAGAAACTCACCTACTTAGACCTTTCACGTAATGG
TCTAACTGGTCTCTTGTCTAGAGCTCATTTTGAAGGACTCTCAGAGCTTGTCCACATTAATT
TAGGGAACAATTTACTCAGCGGGAGCCTTCCTGCATATATATTTGAGCTCCCCTCGTTGCAG
CAGCTTTTTCTTTACAGAAATCAATTTGTTGGCCAAGTCGACGAATTTCGCAATGCATCCTC
CTCTCCGTTGGATACAGTTGACTTGACAAACAACCACCTGAATGGATCGATTCCGAAGTCCA
TGTTTGAAATTGAAAGGCTTAAGGTGCTCTCACTTTCTTCCAACTTCTTTAGAGGGACAGTG
CCCCTTGACCTCATTGGGAGGCTGAGCAACCTTTCAAGACTGGAGCTTTCTTACAATAACTT
GACTGTTGATGCAAGTAGCAGCAATTCAACCTCTTTCACATTTCCCCAGTTGAACATATTGA
AATTAGCGTCTTGTCGGCTGCAAAAGTTCCCCGATCTCAAGAATCAGTCATGGATGATGCAC
TTAGACCTTTCAGACAACCAAATATTGGGGGCAATACCAAATTGGATCTGGGGAATTGGTGG
TGGAGGTCTCACCCACCTGAATCTTTCATTCAATCAGCTGGAGTACGTGGAACAGCCTTACA
CTGCTTCCAGCAATCTTGTAGTCCTTGATTTGCATTCCAACCGTTTAAAAGGTGACTTACTA
ATACCACCTTGCACTGCCATCTATGTGGACTACTCTAGCAATAATTTAAACAATTCCATCCC
AACAGATATTGGAAAGTCTCTTGGTTTTGCCTCCTTTTTCTCGGTAGCAAACAATGGCATTA
CTGGAATAATTCCTGAATCCATATGCAACTGCAGCTACCTTCAAGTTCTTGATTTCTCTAAC
AATGCCTTGAGTGGAACAATACCACCATGTCTACTGGAATATAGTACAAAACTTGGAGTGCT
GAATCTTGGGAACAATAAACTCAATGGTGTTATACCAGATTCATTTTCAATTGGTTGTGCTC
TACAAACATTAGACCTCAGTGCGAATAACTTACAAGGCAGGCTGCCAAAATCGATTGTGAAT
TGTAAGTTGTTGGAGGTCCTGAATGTTGGAAATAACAGACTTGTTGATCATTTCCCATGCAT
GTTGAGGAACTCAAACAGTCTGAGGGTCCTAGTCTTGCGCTCCAATAAATTCTATGGAAATC
TTATGTGTGATGTAACCAGAAATAGCTGGCAGAATCTCCAGATCATAGATATAGCTTCCAAC
AACTTCACTGGTGTGTTGAATGCAGAATTCTTTTCAAATTGGAGAGGAATGATGGTTGCAGA
TGATTACGTGGAGACAGGACGCAATCATATCCAGTATGAGTTCTTACAACTAAGT

Fig. 5B

AAATTGTACTATCAGGACACAGTGACATTAACCATCAAAGGCATGGAGCTGGAGCTTGTGAA
GATTCTCAGGGTCTTCACATCTATTGATTTCTCTTCCAATAGATTTCAAGGAGCGATACCAG
ATGCTATCGGGAATCTCAGCTCACTTTATGTTCTGAATCTGTCACACAATGCCCTTGAGGGA
CCAATCCCAAAATCGATTGGGAAGCTACAAATGCTTGAATCACTAGACCTGTCAACAAACCA
CCTGTCCGGGGAGATCCCATCAGAGCTTGCAAGTCTCACATTCTTAGCAGCTTTGAACTTAT
CGTTCAACAAATTGTTTGGCAAAATTCCATCAACTAATCAGTTTCAAACATTCTCAGCAGAT
TCCTTTGAAGGAAACAGTGGCCTATGCGGGCTCCCTCTCAACAACAGTTGTCAAAGCAATGG
CTCAGCCTCAGAGTCCCTGCCTCCACCAACTCCGCTACCAGACTCAGATGATGAATGGGAGT
TCATTTTTGCAGCAGTTGGATACATAGTAGGGGCAGCAAATACTATTTCAGTTGTGTGGTTT
TACAAGCCAGTGAAGAAATGGTTTGATAAGCATATGGAGAAATGCTTGCTTTGGTTTTCAAG
AAAGTGA

Fig. 6A

Ve1.2 cDNA

ATGAAAATGATGGCAACTCTGTACTTCCCTATGGTTCTCTTGATTCCCTCGTTTCAAATCTT
ATCAGGATACCACATTTTCTTGGTTTCCTCTCAATGCCTTGACGATCAAAAGTCATTGTTGC
TGCAGTTTAAGGGAAGCCTCCAATATGATTCTACTTTGTCAAAGAAATTGGCAAAATGGAAC
GACATGACAAGTGAATGTTGCAATTGGAATGGGGTTACATGCAATCTCTTTGGTCATGTCAT
CGCTTTGGAACTGGATGATGAGACTATTTCTAGTGGAATTGAGAATTCTAGTGCACTTTTCA
GTCTTCAATATCTTGAGAGCCTAAATTTGGCTGACAACATGTTCAATGTTGGCATACCAGTT
GGTATAGACAACCTCACAAACTTGAAGTACCTGAATTTATCCAATGCTGGTTTTGTCGGGCA
AATTCCTATAACATTATCAAGATTAACAAGGCTAGTTACTCTTGATCTCTCAACTATTCTCC
CTTTTTTTGATCAGCCACTTAAACTTGAGAATCCCAATTTGAGTCATTTCATTGAGAACTCA
ACAGAGCTTAGAGAGCTTTACCTTGATGGGGTTGATCTTTCGTCTCAGAGGTCTGAGTGGTG
TCAATCTTTATCTTTACATTTGCCTAACTTGACCGTTTTGAGCTTGCGTGATTGTCAAATTT
CAGGCCCTTTGGATGAATCACTTACTAAGCTTCACTTTCTCTCTTTTGTCCAACTTGACCAG
AACAATCTCTCTAGCACAGTTCCTGAATATTTTGCCAATTTCTCGAACTTGACTACATTGAC
CCTGGGCTCTTGTAATCTACAGGGAACATTTCCTGAAAGAATCTTTCAGGTATCAGTTTTAG
AGAGTTTGGACTTGTCAATTAACAAGTTGCTTCGTGGTAGTATTCCAATTTTTTTCCGAAAT
GGATCTCTGAGGAGGATATCACTAAGCTACACCAACTTTTCCGGTTCATTACCAGAGTCCAT
TTCGAACCATCAAAATCTATCCAGGTTAGAGCTTTCTAATTGCAATTTCTATGGATCAATAC
CTTCCACAATGGCAAACCTTAGAAATCTTGGTTATTTGGATTTCTCCTTCAACAATTTCACT
GGTTCTATCCCATATTTTCGACTGTCCAAGAAACTCACCTACTTAGACCTTTCACGTAATGG
TCTAACTGGTCTCTTGTCTAGAGCTCATTTTGAAGGACTCTCAGAGCTTGTCCACATTAATT
TAGGGAACAATTTACTCAGCGGGAGCCTTCCTGCATATATATTTGAGCTCCCCTCGTTGCAG
CAGCTTTTTCTTTACAGAAATCAATTTGTTGGCCAAGTCGACGAATTTCGCAATGCATCCTC
CTCTCCGTTGGATACAGTTGACTTGACAAACAACCACCTGAATGGATCGATTCCGAAGTCCA
TGTTTGAAATTGAAAGGCTTAAGGTGCTCTCACTTTCTTCCAACTTCTTTAGAGGGACAGTG
CCCCTTGACCTCATTGGGAGGCTGAGCAACCTTTCAAGACTGGAGCTTTCTTACAATAAGTT
GACTGTTGATGCAAGTAGCAGCAATTCAACCTCTTTCACATTTCCCCAGTTGAACATATTGA
AATTAGCGTCTTGTCGGCTGCAAAAGTTCCCCGATCTCAAGAATCAGTCATGGATGATGCAC
TTAGACCTTTCAGACAACCAAATATTGGGGGCAATACCAAATTGGATCTGGGGAATTGGTGG
TGGAGGTCTCACCCACCTGAATCTTTCATTCAATCAGCTGGAGTACGTGGAACAGCCTTACA
CTGCTTCCAGCAATCTTGTAGTCCTTGATTTGCATTCCAACCGTTTAAAAGGTGACTTACTA
ATACCACCTTGCACTGCCATCTATGTGAACTACTCTAGCAATAATTTAAACAATTCCATCCC
AACAGATATTGGAAAGTCTCTTGGTTTTGCCTCCTTTTTCTCGGTAGCAAACAATGGCATTA
CTGGAATAATTCCTGAATCCATATGCAACTGCAGCTACCTTCAAGTTCTTGATTTCTCTAAC
AATGCCTTGAGTGGAACAATACCACCATGTCTACTGGAATATAGTACAAAACTTGGAGTGCT
GAATCTTGGGAACAATAAACTCAATGGTGTTATACCAGATTCATTTTCAATTGGTTGTGCTC
TACAAACATTAGACCTCAGTGCGAATAACTTACAAGGCAGGCTGCCAAAATCGATTGTGAAT
TGTAAGTTGTTGGAGGTCCTGAATGTTGGAAATAACAGACTTGTTGATCATTTCCCATGCAT
GTTGAGGAACTCAAACAGTCTGAGGGTCCTAGTCTTGCGCTCCAATAAATTCTATGGAAATC
TTATGTGTGATGTAACCAGAAATAGCTGGCAGAATCTCCAGATCATAGATATAGCTTCCAAC
AACTTCACTGGTGTGTTGAATGCAGAATT TTTTCAAATTGGAGAGGAATGATGGTTGCAGA
TGATTACGTGGAGACAGGACGCAATCATATCCAGTATGAGTTCTTACAACTAAGT

Fig. 6B

AAATTGTACTATCAGGACACAGTGACATTAACCATCAAAGGCATGGAGCTGGAGCTTGTGAA
GATTCTCAGGGTCTTCACATCTATTGATTTCTCTTCCAATAGATTTCAAGGAGCGATACCAG
ATGCTATCGGGAATCTCAGCTCACTTTATGTTCTGAATCTGTCACACAATGCCCTTGAGGGA
CCAATCCCAAAATCGATTGGGAAGCTACAAATGCTTGAATCACTAGACCTGTCAACAAACCA
CCTGTCCGGGGAGATCCCATCAGAGCTTGCAAGTCTCACATTCTTAGCAGCTTTGAACTTAT
CGTTCAACAAATTGTTTGGCAAAATTCCATCAACTAATCAGTTTCAAACATTCTCAGCAGAT
TCCTTTGAAGGAAACAGTGGCCTATGCGGGCTCCCTCTCAACAACAGTTGTCAAAGCAATGG
CTCAGCCTCAGAGTCCCTGCCTCCACCAACTCCGCTACCAGACTCAGATGATGAATGGGAGT
TCATTTTTGCAGCAGTTGGATACATAGTAGGGGCAGCAAATACTATTTCAGTTGTGTGGTTT
TACAAGCCAGTGAAGAAATGGTTTGATAAGCATATGGAGAAATGCTTGCTTTGGTTTTCAAG
AAAGTGATTATTAAACCCATAAATAATGAGTTTATTCTTGGAGTGTTTTGTTTTAAATAAAC
AACAGGATAAGGAAAATCAAGTTAATAAGCTCGCAGAACATGATTGTTATTTCCTTTGATGA
ATGTATACAATTTTCAATATTGGTTCTTCAACCATAACCGCAGGCTAACTGTCAGTTGTTGG
AAGTCCTGAATTTTGGAAATGACATACATTTTTATAGTTTC

Fig. 7

Ve 1.2 AA from cDNA

A MKMMAATLYFPMVLLIPSFQILSGYHIFLV

B MTSECCNWNGVTCNLFGHVIALELDDETISSGIE
NSSALFSLQYLESLNLADNMFNVGIP
VGIDNLTNLKYLNLSNAGFVGQIP
ITLSRLTRLVTLDLSTILPFFDQP
LKLENPNLSHFIENSTELRELYLDGVDLSSQRS
EWCQSLSLHLPNLTVLSLRDCQISGPLD
ESLTKLHFLSFVQLDQNNLSSTVP
EYFANFSNLTTLTLGSCNLQGTFP
ERIFQVSVLESLDLSINKLLRGSIP
IFFRNGSLRRISLSYTNFSGSLP
ESISNHQNLSRLELSNCNFYGSIP
STMANLRNLGYLDFSFNNFTGSIP
YFRLSKKLTYLDLSRNGLTGLLS
RAHFEGLSELVHINLGNNLLSGSLP
AYIFELPSLQQLFLYRNQFVGQVD
EFRNASSSPLDTVDLTNNHLNGSIP
KSMFEIERLKVLSLSSNFFRGTVP
LDLIGRLSNLSRLELSYNKLTVDAS
SSNSTSFTFPQLNILKLASCRLQKFPD
LKNQSWMMHLDLSDNQILGAIP
NWIWGIGGGGLTHLNLSFNQLEYVEQ
PYTASSNLVVLDLHSNRLKGDLLIP
PCTAIYVNYSSNNLNNSIPTDIG
KSLGFASFFSVANNGITGIIP
ESICNCSYLQVLDFSNNALSGTIP
PCLLEYSTKLGVLNLGNNKLNGVIP
DSFSIGCALQTLDLSANNLQGRLP
KSIVNCKLLEVLNVGNNRLVDHFP
CMLRNSNSLRVLVLRSNKFYGNLM
CDVTRNSWQNLQIIDIASNNFTGVLN
AEFFSNWRGMMVADDYVETGRNHIQ
YEFLQLSKLYYQDTVTLTIKGMELELVKI
LRVFTSIDFSSNRFQGAIP
DAIGNLSSLYVLNLSHNALEGPIP
KSIGKLQMLESLDLSTNHLSGEIP
SELASLTFLAALNLSFNKLFGKIP
STNQFQTFSADSFEGNSGLCGLPLNNSCQSNGSA

E SESLPPPTPLPDSDDEWE

C FIFAAVGYIVGAANTISVVWF

D YKPVKKWFDKHMEKCLLWFSRK

Fig. 8

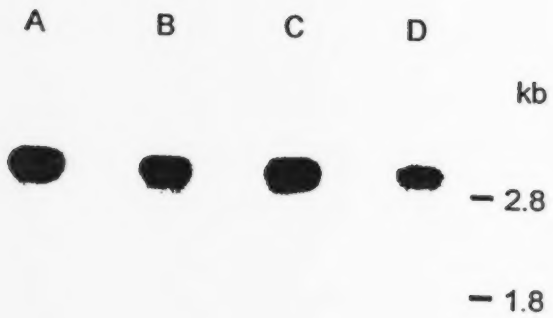

Fig. 9A

Sequence Alignment of Ve1.1 and Ve1.2 AA sequences from cDNA

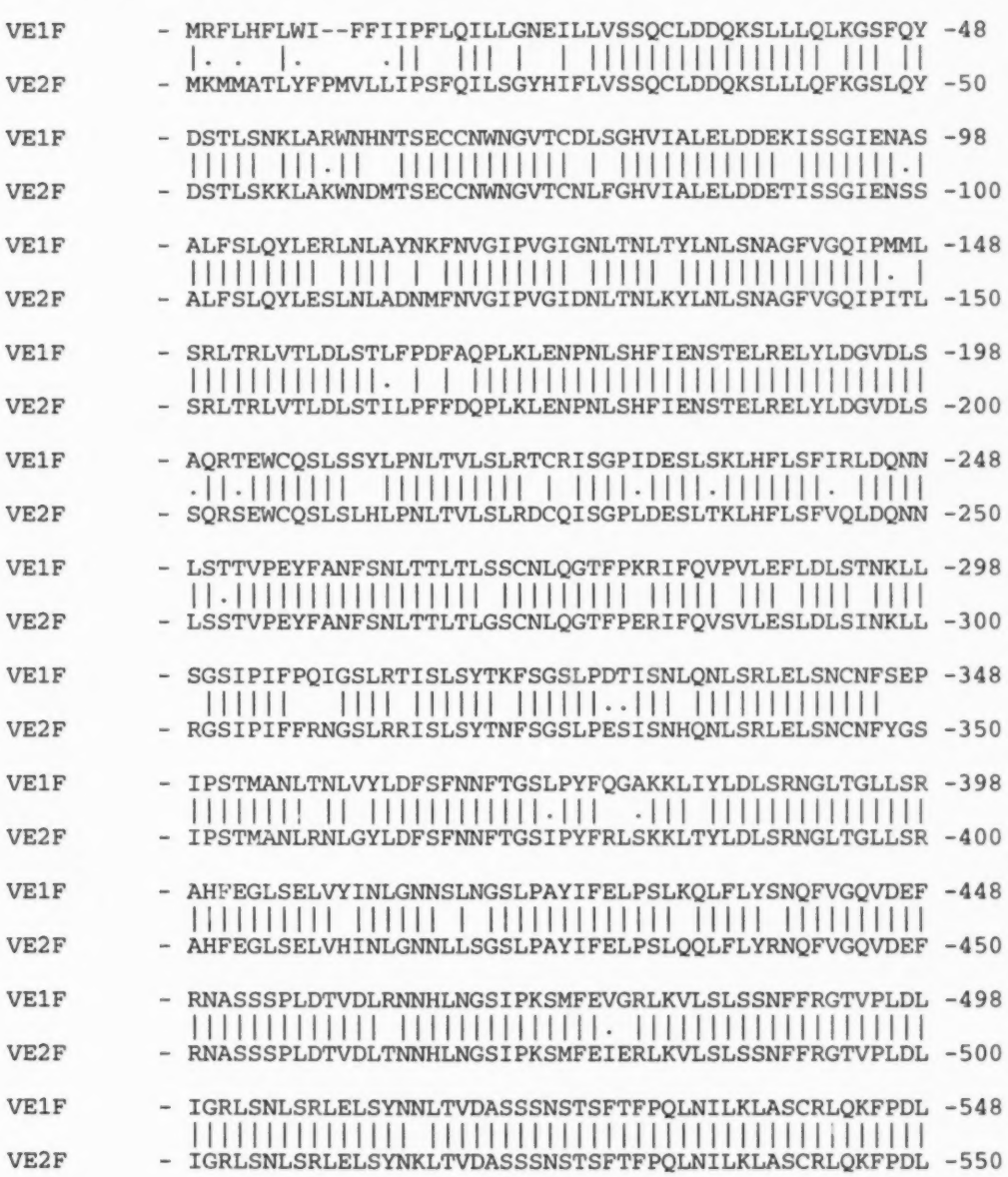
VE1F - MRFLHFLWI--FFIIPFLQILLGNEILLVSSQCLDDQKSLLLQLKGSFQY -48
VE2F - MKMMATLYFPMVLLIPSFQILSGYHIFLVSSQCLDDQKSLLLQFKGSLQY -50
VE1F - DSTLSNKLARWNHNTSECCNWNGVTCDLSGHVIALELDDEKISSGIENAS -98
VE2F - DSTLSKKLAKWNDMTSECCNWNGVTCNLFGHVIALELDDETISSGIENSS -100
VE1F - ALFSLQYLERLNLAYNKFNVGIPVGIGNLTNLTYLNLSNAGFVGQIPMML -148
VE2F - ALFSLQYLESLNLADNMFNVGIPVGIDNLTNLKYLNLSNAGFVGQIPITL -150
VE1F - SRLTRLVTLDLSTLFPDFAQPLKLENPNLSHFIENSTELRELYLDGVDLS -198
VE2F - SRLTRLVTLDLSTILPFFDQPLKLENPNLSHFIENSTELRELYLDGVDLS -200
VE1F - AQRTEWCQSLSSYLPNLTVLSLRTCRISGPIDESLSKLHFLSFIRLDQNN -248
VE2F - SQRSEWCQSLSLHLPNLTVLSLRDCQISGPLDESLTKLHFLSFVQLDQNN -250
VE1F - LSTTVPEYFANFSNLTTLTLSSCNLQGTFPKRIFQVPVLEFLDLSTNKLL -298
VE2F - LSSTVPEYFANFSNLTTLTLGSCNLQGTFPERIFQVSVLESLDLSINKLL -300
VE1F - SGSIPIFPQIGSLRTISLSYTKFSGSLPDTISNLQNLSRLELSNCNFSEP -348
VE2F - RGSIPIFFRNGSLRRISLSYTNFSGSLPESISNHQNLSRLELSNCNFYGS -350
VE1F - IPSTMANLTNLVYLDFSFNNFTGSLPYFQGAKKLIYLDLSRNGLTGLLSR -398
VE2F - IPSTMANLRNLGYLDFSFNNFTGSIPYFRLSKKLTYLDLSRNGLTGLLSR -400
VE1F - AHFEGLSELVYINLGNNSLNGSLPAYIFELPSLKQLFLYSNQFVGQVDEF -448
VE2F - AHFEGLSELVHINLGNNLLSGSLPAYIFELPSLQQLFLYRNQFVGQVDEF -450
VE1F - RNASSSPLDTVDLRNNHLNGSIPKSMFEVGRLKVLSLSSNFFRGTVPLDL -498
VE2F - RNASSSPLDTVDLTNNHLNGSIPKSMFEIERLKVLSLSSNFFRGTVPLDL -500
VE1F - IGRLSNLSRLELSYNNLTVDASSSNSTSFTFPQLNILKLASCRLQKFPDL -548
VE2F - IGRLSNLSRLELSYNKLTVDASSSNSTSFTFPQLNILKLASCRLQKFPDL -550

Fig. 9B

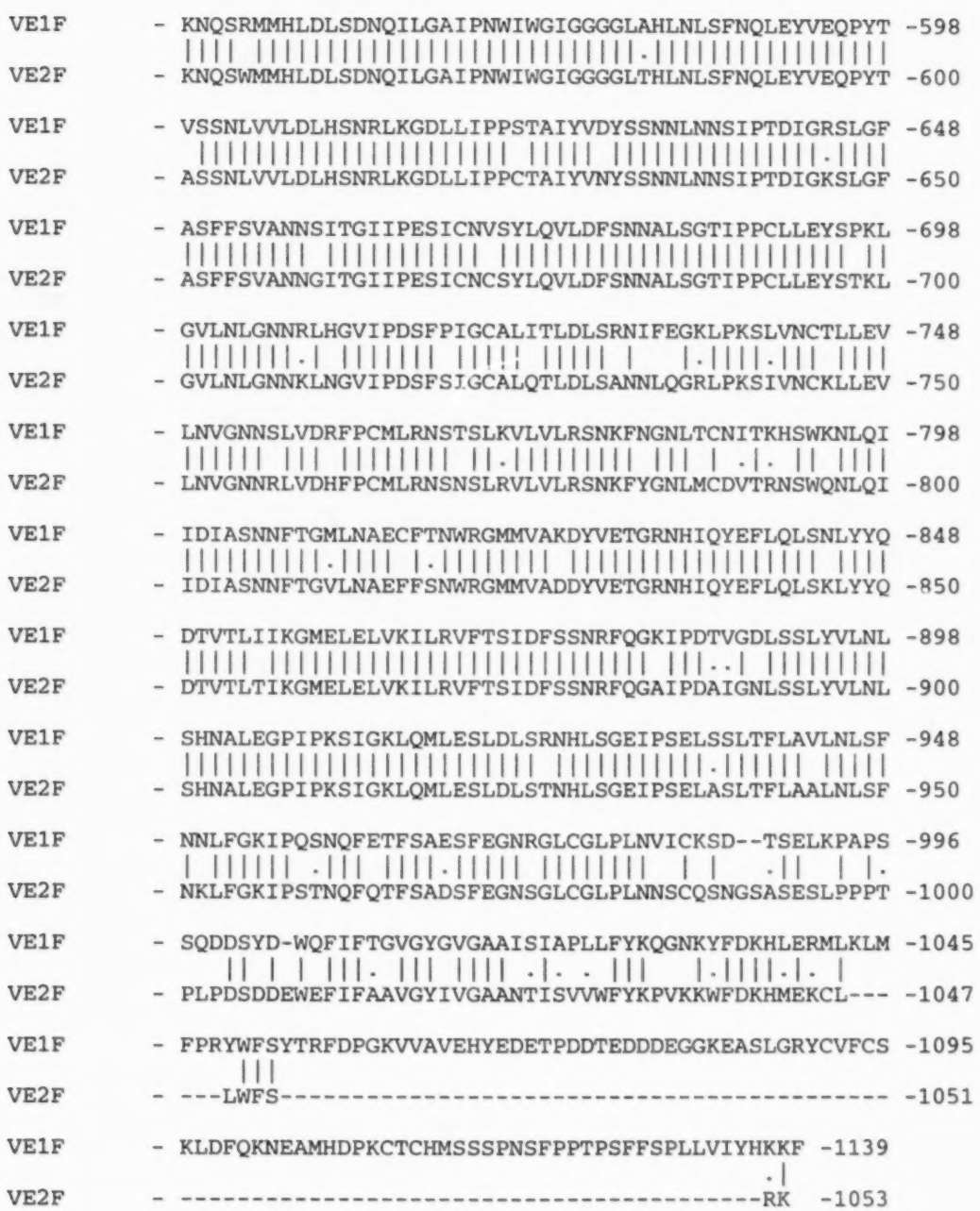
VE1F - KNQSRMMHLDLSDNQILGAIPNWIWGIGGGGLAHLNLSFNQLEYVEQPYT -598
VE2F - KNQSWMMHLDLSDNQILGAIPNWIWGIGGGGLTHLNLSFNQLEYVEQPYT -600
VE1F - VSSNLVVLDLHSNRLKGDLLIPPSTAIYVDYSSNNLNNSIPTDIGRSLGF -648
VE2F - ASSNLVVLDLHSNRLKGDLLIPPCTAIYVNYSSNNLNNSIPTDIGKSLGF -650
VE1F - ASFFSVANNSITGIIPESICNVSYLQVLDFSNNALSGTIPPCLLEYSPKL -698
VE2F - ASFFSVANNGITGIIPESICNCSYLQVLDFSNNALSGTIPPCLLEYSTKL -700
VE1F - GVLNLGNNRLHGVIPDSFPIGCALITLDLSRNIFEGKLPKSLVNCTLLEV -748
VE2F - GVLNLGNNKLNGVIPDSFSIGCALQTLDLSANNLQGRLPKSIVNCKLLEV -750
VE1F - LNVGNNSLVDRFPCMLRNSTSLKVLVLRSNKFNGNLTCNITKHSWKNLQI -798
VE2F - LNVGNNRLVDHFPCMLRNSNSLRVLVLRSNKFYGNLMCDVTRNSWQNLQI -800
VE1F - IDIASNNFTGMLNAECFTNWRGMMVAKDYVETGRNHIQYEFLQLSNLYYQ -848
VE2F - IDIASNNFTGVLNAEFFSNWRGMMVADDYVETGRNHIQYEFLQLSKLYYQ -850
VE1F - DTVTLIIKGMELELVKILRVFTSIDFSSNRFQGKIPDTVGDLSSLYVLNL -898
VE2F - DTVTLTIKGMELELVKILRVFTSIDFSSNRFQGAIPDAIGNLSSLYVLNL -900
VE1F - SHNALEGPIPKSIGKLQMLESLDLSRNHLSGEIPSELSSLTFLAVLNLSF -948
VE2F - SHNALEGPIPKSIGKLQMLESLDLSTNHLSGEIPSELASLTFLAALNLSF -950
VE1F - NNLFGKIPQSNQFETFSAESFEGNRGLCGLPLNVICKSD--TSELKPAPS -996
VE2F - NKLFGKIPSTNQFQTFSADSFEGNSGLCGLPLNNSCQSNGSASESLPPPT -1000
VE1F - SQDDSYD-WQFIFTGVGYGVGAAISIAPLLFYKQGNKYFDKHLERMLKLM -1045
VE2F - PLPDSDDEWEFIFAAVGYIVGAANTISVVWFYKPVKKWFDKHMEKCL--- -1047
VE1F - FPRYWFSYTRFDPGKVVAVEHYEDETPDDTEDDDEGGKEASLGRYCVFCS -1095
VE2F - ---LWFS---------- -1051
VE1F - KLDFQKNEAMHDPKCTCHMSSSPNSFPPTPSFFSPLLVIYHKKF -1139
VE2F - ----------RK -1053

Fig. 10

Vc Partial Genomic DNA Sequence

CATGATTACGCCAAGCTATTTAGGGACATATAGAATACTCAAGCTATGCATCCAACGCGTGG
GGGAGCTCTCCCATATGGTCGACCTGCAGGCGNCCGCGAATTCACTAGTGATTAATTCACTC
AACGGGAGCCTTCCTGCATGTATCTTTGAGCTTCCCTCCTTGCAGACGCTTTTACTTAACAG
CAATCAATTTGTTGGCCAAGTCAACCATTTTCACAATGCATCCTCCTTTCTCGATGAAATTG
ATTTGAGCAACAACCAACTGAATGGTTCAATTCCCAAGTCCATGTTTGACGTTGGGAGGCTT
AAGGTTCTCTCACTTTCTTCCAATTTCTTTAGCGGAACAGTACCCCTTGACCTCATTGGGAA
GCTGAGCAATCTTTCACGACTGGAGCTTTCTTACAATAACTTGACTGTTGATGCAAGTAGCA
GTAATTCAGACTCTTTCACATTTCCCCAGTTGAACATATTGAAACTAGCTTCGTGTCGGCTG
CAAAAGTTTCCTGATCTTAAAAATCAGTCAAGGATGATCCAATTAGACCTTTCTGACAACAA
AATACTGGGGGCAATACCAAATTGGATTTGGCGAATAGGTAACGGAGCTCTGAGTCACCTGA
ATCTTTCTTTCAATCAGTTGGAGTACGTGGAACAGCCTTACAATGTTTCCAGATATCTTGTC
GTCCTTGACTTGCATTCCAATAAGCTAAAGGGTGACCTACCAATTCCACCTTCCTTTGCTGC
ATATTTGGACTACTCGAGCAATAATTTCAGCAATTCCATCCCACTAGATATTGGCAATTATC
TTGGTTTTGCCTCCTTTTTCTCGGTAGCAAACAATGGCATTACTGGAAGAATTCCCGAATCC
ATATGCAATGTCAGCTACCTTCAAGTTCTTGATTTCTCTAACAATGCCTTGAAATCGAATTC
CCGCGGCCGCCATGGCGGCCGGGAGCATGCGACGTCGGGCCCAATTCGCCCT

Fig. 11A

Sequence Alignment Ve1.2 and Vc

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744  NCKLLEVLNVGNNRLVDHFPCMLRNSNSLRVLVLRSNKFYGNLMCDVTRNSWQNLQIIDI 803
     NCKLLEVLNVGNN+LVD FPCMLRNS SL+VLVLRSNKF GNL C++TRNSW+NLQIIDI
1    NCKLLEVLNVGNNKLVDCFPCMLRNSTSLKVLVLRSNKFNGNLTCNITRNSWKNLQIIDI 60

804  ASNNFTGVLNAEFFSNWRGMMVADDYVETGRNHIQYEFLQLSKLYYQDTVTLTIKGMELE 863
     ASNNFTG+LNAE FSNWRGMMVADDYVETGRNHIQY+F QLS LYYQDTVTLTIKGMELE
61   ASNNFTGMLNAECFSNWRGMMVADDYVETGRNHIQYKFFQLSNLYYQDTVTLTIKGMELE 120

864  LVKILRVFTSIDFSSNRFQGAIPDAIGNLSSLYVLNLSHNALEGPIPKSIGKLQMLESLD 923
     LVKILRVFTSIDFSSNRFQG IPD  G+LSSLYVLNLSHNALEGPIPKSIGKLQMLESLD
121  LVKILRVFTSIDFSSNRFQGMIPDTFGHLSSLYVLNLSHNALEGPIPKSIGKLQMLESLD 180

924  LSTNHLSGEIPSELASLTFLAALNLSFNKLFGKIPSTNQFQTFSADSFEGNSGLCGLPLN 983
     LS NHLSGEIPSELASLTFLAALNLSFNKLFGKIPSTNQFQTFSADSFEGN GLCGLPLN
181  LSRNHLSGEIPSELASLTFLAALNLSFNKLFGKIPSTNQFQTFSADSFEGNRGLCGLPLN 240

984  NSCQSNGSAXXXXXXXXXXXXXXXXXWEFIFAAVGYIVGAAN 1024
      +C+S+                    W+FIFAAVGYIVGAAN
241  VTCKSDTPELKPAPSFQEDSDSDYEWKFIFAAVGYIVGAAN 281
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